

## *Clostridium difficile*: Clinical Disease and Diagnosis

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### INTRODUCTION

Although the clinical syndrome of pseudomembranous colitis has been recognized for nearly a century, there have been significant advances in understanding the etiology and in treating and diagnosing the disease in the last 15 years. Initial observations of animal systems demonstrated that hamsters and guinea pigs often respond to the administration of antibiotics by developing a fatal enterocolitis (79, 119, 130, 166, 249). Further studies showed that sterile fecal filtrates from moribund animals alter the morphology of cultured mammalian cells. This effect was prevented when the fecal filtrate was preincubated with *Clostridium sordellii* antiserum, suggesting that a microbial toxin was involved in the induction of disease (28, 230–232).

Advances in knowledge about the etiology of pseudomembranous colitis in humans have paralleled studies in hamsters and guinea pigs. Sterile fecal filtrates from patients receiving antibiotics also induced morphological changes, which could be neutralized by *C. sordellii* antiserum, in a variety of cultured mammalian cells (74, 155). Further studies identified *Clostridium difficile* as the etiologic agent of pseudomembranous colitis in humans (25, 101, 156). The microorganism produces at least two lethal toxins (enterotoxin designated toxin A and cytotoxin designated toxin B) that immunologically cross-react with *C. sordellii* toxins (hemorrhagic toxin designated toxin HT and lethal cytotoxin designated toxin LT) and can be neutralized by *C. sordellii* antiserum (13, 33, 184, 185, 214). Both HT and LT toxins of *C. sordellii* are very similar to the A and B toxins, respectively, of *C. difficile* (185). The production of these toxins by *C. difficile* plays a major role in the induction of enteric disease (1, 24, 35, 41). This finding has led to numerous

efforts to develop methods of detecting and diagnosing the human disease.

Despite the wealth of knowledge related to the diagnosis of *C. difficile*-associated disease, many aspects of clinical tests and assays continue to be controversial. The purpose of this review is twofold: to understand the clinical perspectives of *C. difficile*-associated enteric disease and to assess the concepts of current diagnostic methods. For specific information on the characteristics of *C. difficile* toxins, virulence factors, and treatment of the disease, the reader is referred to several prominent publications or review papers (20, 22, 45, 47, 51, 54, 55, 68, 87, 97, 169, 187).

### HISTORICAL PERSPECTIVES

Pseudomembranous colitis was first described in 1893 by Finney (86). His patient, Mary G., had a “diphtheritic colitis” that manifested as plaquelike membranes in the stomach and lower small bowel. The latter displayed hemorrhages and was covered by a granular exudate. A fibrinous membrane and gray appearance were observed in the large bowel. Reports of this syndrome appeared in the literature with diminishing frequency for several decades. In 1939, a review of 40 fatal cases by Penner and Bernheim (207) suggested that the lesions developed from intestinal anoxia during states of hypotension. A retrospective study on postoperative enterocolitis at the Mayo Clinic from 1925 to 1952 by Pettet et al. (210) revealed a rate of approximately three cases per year. Although an association between antibacterial agents and pseudomembranous colitis was considered, an analysis of the incidence of disease before and after the discovery of antibiotics did not show a statistically significant increase (210).

In numerous early studies, *Staphylococcus aureus* appeared to be the etiologic agent (5, 128, 283). Although staphylococci could be isolated from the stools of patients with high fever and abdominal distress, several patients from

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whom the microorganism had been isolated showed no intestinal lesions at autopsy (67). Further, it was difficult to induce staphylococcal enteritis in animal systems (121, 141, 296). However, with the increased use of antibiotics, the incidence of pseudomembranous colitis continued to increase (128). Studies by Tedesco et al. (264) showed that 21% of patients reported diarrhea as a complication of clindamycin therapy. One-half of these patients were confirmed by sigmoidoscopic examination to have pseudomembranous colitis, prompting the Food and Drug Administration to require a warning on the package insert for clindamycin. In other studies, clindamycin and ampicillin were implicated in a significant number of cases that resulted in pseudomembranous colitis (65, 85, 106, 256). A cooperative study comparing the rates of diarrhea following the administration of clindamycin and ampicillin demonstrated rates of 10 to 20 and 0 to 10%, respectively (115). The occurrence of confirmed pseudomembranous colitis during this cooperative study was 2% in patients treated with clindamycin and 0.3% in those receiving ampicillin treatment.

The etiology of antibiotic-associated diarrhea and pseudomembranous colitis was actively debated in the medical literature for several decades. Because of wide variations in the suspected etiology, no single factor was firmly established as the cause. The rate of incidence of antibiotic-associated diarrhea and pseudomembranous colitis varied among different studies, and the diseases displayed multiple pathological entities ranging from severe and life-threatening to mild and self-limited (26, 158, 165, 213). Published concepts concerning the cause of antibiotic-associated diarrhea and pseudomembranous colitis ranged from a direct effect of the antibiotic or a metabolite of the antibiotic on the intestinal mucosa (213) to changes in the intestinal microflora or a localized Schwartzmann reaction (125, 221). In several studies, viruslike particles were visualized by electron microscopy of intestinal biopsy samples from patients with pseudomembranous colitis (111, 165, 253). However, viruses have never been propagated from patients with the disease. It has since become apparent, through studies involving animal models and the toxicity of fecal filtrates for cultured mammalian cells, that the major etiology of antibiotic-associated diarrhea and pseudomembranous colitis is *C. difficile* (17, 20, 64, 74, 80, 84, 131, 140, 195, 206, 222).

The initial report of a bacterial toxin in the feces of a patient involved a young girl who developed pseudomembranous colitis after a course of oral penicillin for acute pharyngitis (154). During the course of investigation, virologic studies of fecal specimens completed with cultured HeLa, rhesus monkey kidney, and human embryonic lung fibroblastic cells demonstrated a characteristic cytopathic effect that could not be propagated upon subculture. These results suggested the presence of a bacterial toxin, although a specific microorganism was not implicated. Cytotoxic stool filtrates were obtained from several other patients with pseudomembranous colitis; stool samples from convalescent patients and healthy individuals were negative when tested for cytopathic effect on cultured mammalian cells. A simultaneous report by Rifkin et al. (231) on the presence of a fecal toxin in the fecal filtrates from two patients with pseudomembranous colitis appeared to confirm and extend this early observation. The sterile toxic fecal filtrates from these patients induced a lethal enterocolitis in hamsters following intraperitoneal challenge, increased intravascular permeability when tested in rabbit skin, and altered the morphology of cultured mammalian cells. The toxic factor

could be neutralized by *C. sordellii* and polyvalent gas gangrene antisera, inactivated by heat, and destroyed by the enzyme pronase (155, 227, 235). The additional finding that oral vancomycin therapy was followed by clinical improvement was further support for the concept that pseudomembranous colitis was caused by a toxic factor produced by a microorganism (21, 27, 44).

Other studies supported the finding that a toxic factor found in the feces of patients with pseudomembranous colitis could be neutralized by polyvalent clostridial antiserum and specifically with antiserum directed against *C. sordellii* (4, 49, 155, 164). These observations led to the premature conclusion that *C. sordellii* was the pathogen responsible for antibiotic-associated pseudomembranous colitis (152). However, additional studies demonstrated that *C. sordellii* could not be isolated from patients with the disease (169, 291).

Quantitative cultures of feces from patients confirmed to have pseudomembranous colitis resulted in the recovery of four clindamycin-resistant clostridial isolates (24). These microorganisms produced a cytotoxin that demonstrated a positive cytopathic response when tested with cultured mammalian cells. The isolates were identified biochemically and culturally as *C. difficile* (48, 193, 251). The realization that *C. difficile* produced a toxic factor that could be neutralized by *C. sordellii* antiserum provided the connecting link between culture data implicating *C. difficile* and neutralization studies implicating *C. sordellii* (17, 25, 50, 93, 100, 101).

#### CLINICAL AND PATHOLOGICAL FEATURES OF DISEASE

The clinical and pathological features of disease caused by *C. difficile* indicate that it is difficult to distinguish from other intestinal diseases, including ulcerative colitis, chronic inflammatory bowel disease, and Crohn's disease (30, 92, 272). In an analysis of early reports of pseudomembranous colitis, Bartlett and Gorbach (26) observed that the clinical course typically showed an abrupt onset of nonspecific intestinal symptoms on the second to fifth postoperative day followed by hypotension and death within a few days. Although involvement of the small bowel was frequent and extensive, pseudomembranes could be found throughout all segments of the gastrointestinal tract, including the esophagus and stomach, and toxic megacolon could develop (58, 81, 205).

The symptoms of *C. difficile*-induced enteric disease often occur after 5 to 10 days of antimicrobial therapy, although documented symptoms have been demonstrated to occur as early as the first or second day of therapy or as late as 2 to 10 weeks after the offending antimicrobial agent is discontinued (94, 98, 263). A single dose of intravenous antibiotics or the administration of antineoplastic (78) or antiviral (56) agents has also been shown to cause *C. difficile*-associated disease (6).

Approximately 90 to 95% of all patients with *C. difficile*-associated disease have brown or clear watery diarrhea, and the remaining 5 to 10% have bloody diarrhea. Eighty-five percent of patients with pseudomembranous colitis appear to have mucus in their feces (264). Temperature is elevated and exceeds 101°F (ca. 38°C) in 26 to 66% of patients (102, 196, 262). Leukocytosis is a common clinical feature in the severely ill patient, with leukocyte counts exceeding 15,000 cells per mm<sup>3</sup> in 40 to 50% of patients (196, 262). Several studies have demonstrated that toxin A of *C. difficile* is a potent activator and chemoattractant for human leukocytes,

suggesting an infiltration of the intestinal lamina propria and subsequent release of inflammatory mediators (31, 216, 273). Triadafilopoulos et al. (273) demonstrated that toxin A causes an increased chemotactic response of leukocytes in elderly (>65 years old) subjects compared with young (20 to 35 years old) subjects, which may explain the enhanced inflammatory phenomena observed in the elderly with *C. difficile* disease. These observations may also be related to the presence of fecal leukocytes in patients with pancolitis (19).

The clinical spectrum in patients with *C. difficile*-associated disease may range from a mild diarrhea to a fulminant pancolitis, including toxic megacolon and bowel perforation (23, 205). Complete involvement with pseudomembranous colitis is marked by patchy areas of mucosal inflammation in the intestinal epithelium, with gray to yellow coagula composed of sloughed epithelial cells, leukocytes, and fibrin forming the pseudomembrane. The underlying mucosal surface often reveals well-defined petechial lesions and ulcerations of the mucosa consisting of epithelial necrosis and a marked leukocytic infiltration of the lamina propria (1, 3, 20, 123, 272).

### PEDIATRIC INFECTIONS

A unique aspect of pseudomembranous colitis in the pediatric population is related to the observation that *C. difficile* frequently colonizes infants and young children but without causing signs or symptoms of disease. Studies on the prevalence of *C. difficile* infection in healthy newborn infants and young children have demonstrated acquisition rates of 15 to 63% (34, 35, 73, 118, 153, 178, 228, 252, 258); however, other studies have reported acquisition rates of 0 to 6% (127, 153, 246, 257). These differences in the acquisition rates of *C. difficile* in neonates and young children most likely represent varied degrees of environmental exposure in nurseries or transfer from the hands of hospital staff rather than disparities in the rate of maternal exposure (12). Prevalence of the carrier state has made it difficult to assess the relationship between *C. difficile* colonization and disease in this age group (57).

Isolates of *C. difficile* from infants have been shown to be highly toxic in vitro (36, 279), although between 26 and 69% of isolates have been found to be nontoxic (34, 76, 153, 178, 203, 228, 268). Cooperstock et al. (59) have demonstrated that 40% of healthy infants colonized with *C. difficile* have fecal toxin titers of at least  $10^4$ . Fecal toxin titers are rare in carriers over the age of 2 years. Although bouts of diarrhea are frequent in children who have been given antibiotics, documented cases of pseudomembranous colitis are few (113, 138, 145, 286). However, reports of pseudomembranous colitis in children have been documented (2, 11, 179, 225, 229, 243). Although the reason for the absence of disease progression in neonates with high fecal toxin titers is unclear, Chang et al. (53) have suggested that the lack may be due to a deficiency of cell membrane toxin receptors on neonatal cells.

### EPIDEMIOLOGY

Studies on the rate of intestinal carriage of *C. difficile* by healthy adults in a number of geographical areas have been completed (82, 83, 183, 190). The carriage rates range from 2% in Sweden to 15% in Japan (8, 9, 99, 156, 185, 202). However, comparison of the rates of carriage must consider the method of culture (selective versus nonselective media)

and the method of selection for healthy adults with no prior exposure to antimicrobial agents. Although the rate of spontaneous pseudomembranous colitis following antibiotic therapy is low, several clusters of cases have been reported (136, 194, 212, 226, 264).

The spread of *C. difficile* in four hospitals has been documented by Mulligan et al. (200). Cultures performed on samples taken from rooms occupied by patients with pseudomembranous colitis demonstrated that *C. difficile* was present in toilet areas, floors, and bed handrails; about 33% of all sites tested were culture positive for *C. difficile*. In rooms not occupied by such patients, 1.3% of all sites tested were culture positive. In another study, Kim et al. (146) showed that 19.6% of environmental cultures were positive for *C. difficile* when samples for culture were obtained from the room of a patient with pseudomembranous colitis. The highest rates for positive culture were for samples from around toilets and sinks, on bathroom floors, under patients' beds, and on bedclothes. These rates of isolation support the concept that *C. difficile* is the most common cause of diarrheal disease in the hospital setting.

At least one extraintestinal source of *C. difficile* has been reported by Hafiz et al. (116). In their studies, vaginal specimens from 72% of 108 female patients at a sexually transmitted disease clinic were culture positive. Further, *C. difficile* was isolated from the urethral discharge of each of 42 male patients with nonspecific urethritis. However, subsequent investigations of this issue have failed to confirm these observations (90, 186, 199). The etiologic agent *C. difficile* has been isolated infrequently from other human sources (109, 110, 250). Other environmental sources include marine sediment, soil, sand, mud, and animal dung (39, 117, 124, 186).

### C. *DIFFICILE* TOXINS

Several studies have demonstrated the presence of two toxins, separable by ion-exchange chromatography, in culture filtrates of *C. difficile*. An enterotoxin designated toxin A and a cytotoxin designated toxin B were separated and found to have similar molecular weights but different activities (15, 104, 163, 170, 171, 254, 260, 261, 271). The enterotoxin was found to be 100- to 1,000-fold less cytotoxic than the cytotoxin and demonstrated a fluid response when tested in ligated ileal loops. Polyclonal antibody prepared against partially purified toxin A did not neutralize the activity of toxin B. Several other toxic activities produced by *C. difficile*, including toxin C, have been reported but not well characterized (107, 269, 270).

In other studies, Lyerly et al. used ammonium sulfate precipitation, batch ion-exchange chromatography on DEAE-Sephrose CL-6B, precipitation, and immunoabsorption to isolate toxins A (171) and B (173). Pothoulakis et al. (215) followed similar initial steps but used high-performance liquid chromatography with a Mono-Q anion-exchange column to isolate toxin B. Meador and Tweten (192) also used a Mono-Q column as the final purification step.

The reported molecular weights for native toxins A and B have ranged from 440,000 to 600,000 and 107,000 to 550,000, respectively (10, 14, 15, 129, 130, 148, 235, 259, 265, 290). Banno et al. (14), who used sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, reported a single protein band for toxins A and B with a molecular weight in the range of 190,000 to 200,000. Rihn et al. (234) reported that toxin A had a native molecular weight of 52,000 and could be dissociated with 1% sodium

dodecyl sulfate into two subunits with molecular weights of 41,500 and 16,000. The enzymes RNase,  $\beta$ -galactosidase, and lipase have no effect on either toxin A or toxin B, while pronase, trypsin, and chymotrypsin inactivate both toxins (173).

More recently, von Eichel-Streiber et al. (281, 282) isolated cytotoxin B fragments from a *C. difficile* expression library by using *C. sordellii* lethal toxin antiserum. A partial restriction map of recombinant clones showed that the *toxB* gene is positioned upstream of *utxA* and *toxA* and has a size of 6.9 kb corresponding to a polypeptide size of 250 kDa. A total of 203 N-terminal amino acids of toxins A and B were compared. Sixty-four percent of the residues were homologous, demonstrating the relatedness of toxins A and B. McMillin et al. (191) used the polymerase chain reaction and fluorescent tags to demonstrate that 37 toxic strains of *C. difficile* normally had the genetic composition for toxins A and B and 10 nontoxic strains did not contain detectable toxin determinants. One strain, however, was found to contain the genetic composition for toxins A and B but was not cytotoxic under the conditions tested. The toxin A gene has been demonstrated to encode a single-chain, 308-kDa protein, confirming the molecular weight estimated by polyacrylamide gel electrophoresis (75, 242). The toxin B gene has been cloned and sequenced by Johnson et al. and encodes an apparent 270-kDa protein (16, 134). Comparison of the amino acid sequences of the N-terminal regions of toxins A and B revealed a cluster of 172 hydrophobic and highly conserved amino acids in the center, a sequence of 120 highly conserved amino acids including arginine, cysteine, histidine, methionine, and tryptophan, and an area of 248 less conserved amino acids (282). These data suggest that the genes for toxins A and B may have evolved from a common ancestor. The C-terminal third of toxin A contains 38 contiguous repeat units that are believed to play a crucial role in the binding of toxin to its carbohydrate receptor (75, 274, 300). In other work, toxin B of *C. difficile*, a glucan-binding protein of *Streptococcus mutans*, four glucosyltransferases from *Streptococcus mutans* and *Streptococcus downei*, and several pneumococcus-associated lysins have been demonstrated to contain repeat C-terminal sequences (220, 237, 299, 300). These data will no doubt yield practical information on ligand-receptor interactions, the construction of specific ligand delivery systems, and the development of new techniques and methods for the diagnosis of diseases.

### TOXICITY VERSUS HOST FACTORS IN DISEASE

In spite of the recognition that *C. difficile* toxins are important entities in enteric disease (23, 28, 155), healthy neonates and up to 4% of healthy adults may harbor toxin-producing strains with no ill effects (9, 29, 188, 277, 279, 298). In addition, Gumerlock et al. (114) have demonstrated *C. difficile* in the feces of patients who are toxin negative but have clinical symptomatic disease. Although the clinical implications of these findings are unknown, *C. difficile* is not the only cause of antibiotic-associated diarrhea, and the organism does not appear to cause disease in the absence of toxin production (35, 61, 169). Vernet et al. (276), using gnotobiotic mice monoassociated with various *C. difficile* strains, observed that death was related to the presence of both toxins A and B. However, mice infected with strains of *C. difficile* that produced intermediate amounts of toxins A and B in vitro survived even though high levels of toxin B and nondetectable levels of toxin A were produced in vivo. In another study, toxin B inoculated intragastrically into

animals did not cause a significant effect on health, whereas toxin A resulted in death (174). Although toxin B has an apparent minor pathological effect, it may require the action of toxin A for complete biological expression.

In other investigations, the effect of proteases produced by the host and resident intestinal microflora on the detection of *C. difficile* toxins has been studied (61, 150, 210, 278). Corthier et al. (61) have shown that cecal proteolytic activities in gnotobiotic mice hydrolyze toxin A bound to immunoglobulin G in microtiter plates used in immunoassays. However, the modification of toxin A by intestinal proteases did not affect the ability of the toxin to bind to antibodies used in immunoassays even though lethal activity was reduced 100-fold. The activity of cytotoxin B was not altered by intestinal proteases.

The carriage of nontoxic isolates of *C. difficile* may be related to prior infection with toxic isolates. Corthier and Muller (60) have shown in gnotobiotic mice that stable nontoxic clones can originate from toxic clones. These clones exhibited a protective effect when the mice were challenged with toxic strains of *C. difficile*. Fluit et al. (89) have shown that nontoxic strains of *C. difficile* lack the genes for both toxins A and B. In other studies, Torres (268) has isolated a strain of *C. difficile* that produces toxin B but does not produce a detectable level of toxin A. Gianfrilli et al. (104) have demonstrated that *C. difficile* isolates from patients with pseudomembranous colitis and antibiotic-associated diarrhea produce toxin B, but only approximately 70% of isolates produce a detectable level of toxin A.

A retrospective study by Siegel et al. (248) analyzed the relative yield of ova and parasite examinations and routine stool cultures for outpatients and inpatients and for inpatients in relation to time after admission. During a 3-year period, none of 90 ova and parasite examinations with positive results and only 1 of 191 positive stool cultures were from patients who had submitted stool specimens after 3 days in the hospital. Specimens from these patients represented nearly 50% of the specimens received annually. However, approximately 25% (range, 17 to 33%) of stool specimens were positive for *C. difficile* cytotoxin B. The authors indicate that, at a time when cost containment is considered a priority, low-yield tests such as ova and parasite examinations and routine stool cultures should be reserved for outpatients or inpatients who are admitted with a diarrheal illness. In contrast, tests for the presence of *C. difficile* toxin in clinical samples would appear warranted regardless of admission status (248).

### DIAGNOSIS OF DISEASE

#### Culture of the Etiologic Agent

Symptoms of *C. difficile*-associated disease vary widely, ranging from a mild diarrhea to the colicky or intense abdominal pain accompanied by systemic symptoms such as fever, malaise, and extreme weakness that may signal an acute abdominal emergency. Complications include bowel perforation, toxic megacolon, and indirect endotoxemia. Patient feces often contain excess mucus, and pus or blood may be present (26, 137, 196, 262). Nausea, vomiting, and fever are present in some patients. Leukocytosis is found in 40% of patients, and an abnormally low level of serum albumin is evident in 76% of patients (205).

Although the current or recent use of antibiotics is important as a clue in the diagnosis of *C. difficile*-associated disease, symptoms are not readily distinguishable from those

caused by other enteric pathogens or diseases, including Crohn's disease and ulcerative colitis (92). In addition, no consistent clinical basis on which to differentiate *C. difficile*-associated disease from other diseases has been reported.

Since the clinical presentations of numerous other bowel diseases and irregularities may resemble *C. difficile*-associated disease, diagnosis must be confirmed by colonoscopic or sigmoidoscopic examination and specific laboratory tests. These tests are generally chosen because of their abilities to demonstrate the presence of a pseudomembrane, isolate the offending pathogen rapidly, or detect antigen in clinical specimens. Several bacterial culture systems have proven useful for the isolation of *C. difficile* from clinical specimens. George et al. (99) developed a differential and selective medium containing cycloserine-cefoxitin-fructose and egg yolk (CCFA medium) for the isolation of *C. difficile*. Growth of the microorganism on this medium resulted in a characteristic colonial morphology, which showed a typical green fluorescence under long-wavelength UV light (99). After 48 h of culture under anaerobic conditions, colonies appeared yellowish, flat, circular to irregular, and 4 to 8 mm in diameter. The use of this selective medium has resulted in the isolation of *C. difficile* from 90 to 100% of fecal samples that contain cytotoxin (291, 292). Other studies have demonstrated that approximately 3% of fecal specimens from healthy adults (42, 275), 4 to 62% of fecal specimens from neonates, and 4 to 8% of fecal specimens from children harbor the microorganism (127, 258, 279). In addition, 2 to 11% of fecal specimens from hospitalized adults not on antimicrobial agents (52, 272) and 21 to 46% of fecal specimens from hospitalized adults on antimicrobial agents but without gastrointestinal complaints harbored *C. difficile* (95, 96, 189, 279). The presumptive identification of *C. difficile* was accomplished by using gas-liquid chromatography to screen fecal samples or culture broth for the presence of isocaproic acid or *p*-cresol (66, 108). These substances are also produced by other microorganisms, so their mere presence in patient samples is not specific enough for a definitive clinical diagnosis (43, 66, 105, 159, 161, 177, 208, 211, 217). Definitive identification of *C. difficile* consists of biochemical testing in prerduced anaerobically sterilized broths and end-product analysis by gas-liquid chromatography (126, 255).

Johnson et al. (135) identified *C. difficile* in fecal specimens by using culture-enhanced gas-liquid chromatography. This method detects the metabolic breakdown products of *C. difficile* by gas-liquid chromatography after fecal samples have been incubated in a selective broth medium containing cefoxitin. The sensitivity and specificity of the method were 99.6 and 99.0%, respectively. Low numbers of *C. difficile* in fecal samples are amplified by culture, and the need for subculture to obtain a pure isolate is eliminated. However, the method requires 48 h of incubation and does not identify patients who are carriers of *C. difficile* and have no significant related disease nor does it distinguish toxin-negative from toxin-positive microorganisms.

Several procedures for determining the effectiveness of culture methods for the isolation of *C. difficile* from fecal specimens have been evaluated (38, 182). These methods include the use of either heat shock or ethanol treatment prior to plating and direct plating on three commercially available media. Although ethanol treatment and direct plating on cycloserine-cefoxitin-fructose agar with horse blood (Carr-Scarborough Microbiologicals, Inc., Stone Mountain, Ga.) were the most effective procedures for the isolation of *C. difficile* from fecal specimens, the method also

required 48 h of culture prior to identification. Further, the method did not identify patients who were carriers of *C. difficile* but had no related disease or measure the ability of isolates to produce virulence factors. Diet, the concurrent or recent administration of antibiotics, age, and location may also influence isolation rates (35, 42, 257, 279). It has been reported that as many as 21% of hospitalized patients on antimicrobial therapy may harbor *C. difficile* without any evidence of disease (279). Unfortunately, the high incidence of asymptomatic carriage in healthy individuals precludes the use of culture for routine identification of patients with disease.

Several commercial systems for the identification of anaerobic bacteria have been introduced. These systems include the API ZYM System, Minitex Anaerobe II, API An-Ident System, and RapID ANA System. Although their abilities to identify anaerobes in general were assessed, studies on their specificities for *C. difficile* have given inconsistent results (32, 112, 122, 160, 181). Regrettably, all of these systems require that the organism be in pure culture before use, causing an unavoidable time delay for organism isolation and for diagnosis.

#### Cellular Cytotoxicity Assays

The assay for *C. difficile* cytotoxicity that uses cultured mammalian cells has been widely tested as an aid for diagnosing *C. difficile*-associated disease (74, 233, 236, 266). Gerding et al. (102) have shown that 35 (90%) of 39 endoscopy-positive patients were cytotoxin positive. The cellular cytotoxin assay has also been shown to be positive in ≈97% of all cases of pseudomembranous colitis and in about 27% of all patients with antibiotic-associated diarrhea in whom no demonstrable pseudomembranes were present (17, 25, 29, 81). The cellular cytotoxicity assay is performed by adding a measured amount of sterile (filtered) fecal specimen in the presence and absence of specific antiserum to a cultured monolayer of mammalian cells. After 24 h of incubation, the cultured mammalian cells are observed for cytopathic effect. Since fecal specimens may contain substances that will induce a nonspecific cytopathic effect, specificity is confirmed with a neutralizing high-titered antiserum (278). Detailed procedures can be found in papers by Chang et al. (52), Bartlett (18), Rolfe and Finegold (235), Florin and Thelestam (88), and Maniar et al. (180). In addition, antiserum and lyophilized *C. difficile* toxin are available as a Toxin/Antitoxin Kit (TechLab, Blacksburg, Va.) for laboratories with cell culture facilities. The frequency of cytotoxin detection increases as the severity of symptoms, duration, and pathology increases. A tendency for the titer to increase upon aggravation of symptoms and to decrease with an alleviation of clinical symptoms has been demonstrated (23, 197).

Specimens may be readily posted to the nearest regional medical facility for diagnostic testing when necessary. With the advent of new cell culture techniques, the cellular cytotoxicity assay does not require the availability of a CO<sub>2</sub> incubator or mammalian cell culture facilities, making the test practical for many smaller hospitals and physicians in rural or remote areas. The assay is available as the Toxi-Titer Assay for *C. difficile* Toxin from Bartels Diagnostics (Baxter Healthcare Corp., Bellevue, Wash.). It employs human foreskin fibroblasts and is performed by inoculating the supplied cell culture with a preincubated stool filtrate-antitoxin mixture (169, 201, 301). In a recent comparison with the toxin B cytotoxicity assay (Toxi-Titer), the Bartels *C. difficile* toxin A enzyme immunoassay had a sensitivity of

94%, a specificity of 96%, and positive and negative predictive values of 75 and 99%, respectively (142). Other studies have also compared other cytotoxin assays with various enzyme immunoassays for *C. difficile* (62, 77, 103, 198). Although the low dilution of fecal extract used in the microtiter plate well may lead to an incorrect result, few consistent problems have been noted when the test is completed by a qualified technician.

### Latex Agglutination Tests

The purported detection of *C. difficile* toxin A in fecal specimens by a commercial latex agglutination test has been the subject of much controversy (14, 15, 144). The test kit, first marketed as the Culturette Brand Rapid Latex Test by Marion Laboratories (Kansas City, Mo.), was subsequently purchased and repackaged by Becton Dickinson and Company (Cockeysville, Md.) as the Culturette Brand CDT latex agglutination test. Several studies have reported on the correlation of the latex agglutination test with cytotoxin testing and clinical disease (37, 143, 209, 247, 288, 294, 295). However, the published positive and negative predictive values have varied considerably, ranging from 63 to 98%. Lyerly and Wilkins (176) and Lyerly et al. (167) have reported that the latex agglutination test does not detect toxin A but reacts with a latex-reactive protein that contaminates the commercial antigen preparations used to immunize animals for the production of antibody. The latex-reactive protein copurifies with toxin A and is associated with both toxic and nontoxic isolates of *C. difficile*, other strains of clostridia, *Peptostreptococcus anaerobius*, and *Bacteroides asaccharolyticus* (167). No correlation between the latex-reactive protein and the virulence of *C. difficile* has ever been documented.

In further studies, Lyerly et al. (168) analyzed the Culturette Brand CDT latex agglutination test-reactive protein. The gene encoding the protein was cloned and sequenced, and the protein was demonstrated to be an enzyme. The enzyme, glutamate dehydrogenase, reversibly catalyzes the amination of  $\alpha$ -ketoglutarate to glutamate and the deamination of glutamate to  $\alpha$ -ketoglutarate (63). The native enzyme has an aggregate molecular weight of  $\approx 200,000$  and is highly immunogenic. It has been associated with other bacteria and mammalian cells and plays an important role in cellular metabolism. Therefore, latex reagents that use antibodies directed at this enzyme would not be able to distinguish between toxic and nontoxic isolates of *C. difficile* or between *C. difficile* and other microorganisms or mammalian cells. Huovinen et al. (132) have indicated that latex agglutination tests show false-positive reactions and possess a low specificity for *C. difficile*. Sherman et al. (247) concluded that the latex agglutination test is suitable for rapid screening but that positive results require confirmation by other testing methods. Woods and Yam (295) compared the latex agglutination test and the cytotoxin assay. Using 206 stool specimens submitted for the detection of *C. difficile* toxin, they observed no significant difference between the latex agglutination test and the cellular cytotoxicity assay. Peterson et al. (209) tested 161 fresh stool samples and found the latex agglutination test to be superior to the cellular cytotoxicity assay in diagnosing cases of *C. difficile*-associated diarrhea (90 versus 70% positivity, respectively) in patients who had received antimicrobial therapy within 6 weeks of the onset of diarrhea, had a positive colonoscopy for pseudomembranes or an appropriate response to metronidazole or vancomycin therapy, and had no other obvious etiology for the diarrhea.

However, the high incidence of false-positive reactions with the latex agglutination test for the diagnosis of *C. difficile*-associated disease has been well documented (167, 224). In view of false-positive and, in some instances, false-negative reactions, duplicating latex agglutination test results by a second testing method seems counterproductive (132, 238).

In other studies, Kelly et al. (143) compared the Culturette Brand CDT latex agglutination test (Becton Dickinson) with the Meritec *C. difficile* latex test for antigens (Meridian Diagnostics, Cincinnati, Ohio). The Meritec latex agglutination test showed a sensitivity, a specificity, and a correlation of 90, 97, and 92%, respectively, compared with the Culturette Brand CDT test. A sensitivity, a specificity, and a correlation of 77, 93, and 92% were observed for the Meritec latex test compared with the cellular cytotoxicity assay. The Meritec latex test was not as sensitive as the cellular cytotoxicity assay or culture for the detection of *C. difficile*-associated disease.

### Enzyme-Linked Assay Systems

Numerous studies have investigated techniques for the rapid detection of *C. difficile* toxins (40, 46, 62, 69, 70, 77, 91, 103, 142). An enzyme-linked immunosorbent assay for the presence of toxins A and B was used to examine more than 90 isolates of *C. difficile* for toxin production. All toxic isolates appeared to produce both toxins A and B (157, 175). Nguyen et al. (204) used an enzyme-linked immunoassay for the detection of *C. difficile* toxin B in fecal specimens, finding a close correlation with the culture and isolation of *C. difficile*. The assay appeared to be useful as a presumptive test for detection of *C. difficile* toxin B, with the recommendation that positive reactions be confirmed by both the cytotoxin assay and isolation of the microorganism.

Lyerly et al. (175) developed an enzyme-linked immunosorbent assay for the detection of *C. difficile* toxin A. The assay, available commercially through TechLab (VPI Research Park, Blacksburg, Va.), is marketed as the TOX-A-TEST. The test employs two antibodies (an affinity-purified polyclonal antibody and a monoclonal antibody conjugated to horseradish peroxidase) to detect *C. difficile* toxin A. Test limitations include the following: a need for fresh (less than 24-h-old) clinical samples, which is true for all toxin tests; weak inconclusive reactions produced by some specimens, which thus require additional testing; and an inability to detect *C. difficile* toxin B. However, the test correlates well with the cytotoxin assay procedure.

Woods and Iwen (294) compared a dot immunobinding assay, latex agglutination, and the cellular cytotoxicity assay for the diagnosis of *C. difficile*-associated disease. Specimens representing 198 stool samples from 169 patients were evaluated. The results of dot immunobinding assays (*C. diff*-CUBE; Difco Laboratories, Ann Arbor, Mich.) agreed with those of the cellular cytotoxicity assay and the latex agglutination test in 92 and 88% of cases, respectively. Although the dot immunobinding assay detects only *C. difficile* toxin A, Wood and Iwen (294) suggested that it may be useful as a screening test for the diagnosis of *C. difficile*-associated disease. Two drawbacks to the dot immunobinding assay are its inability to detect *C. difficile* toxin B and its nonspecificity for *C. difficile*. The assay has not been approved for in vitro diagnostic use.

Another direct enzyme immunoassay for the detection of *C. difficile* toxin A was studied by DiPersio et al. (71). The immunoassay, Premier *C. difficile* Toxin A (Meridian Diagnostics), was compared with the cellular cytotoxicity assay,



toxigenic culture assay, and latex agglutination. Of 313 fresh stool specimens, 62 were positive by one or more of the tests, but only 22 (35%) were positive by all four tests. Totals of 85.3, 94.1, 79.4, and 58.8% of 34 patients determined to have *C. difficile*-associated disease were positive by the direct immunoassay, cellular cytotoxicity assay, toxigenic culture assay, and latex agglutination test, respectively. Seven patients who had positive latex agglutination tests showed negative results in the direct enzyme immunoassay, cellular cytotoxicity assay, and toxigenic culture assay. In a separate study, the Premier *C. difficile* Toxin A kit was evaluated with 101 fecal specimens (40). Sixty-nine specimens were positive by the cellular cytotoxicity assay and isolation of the microorganism. Of these 69 cases, 49 were positive by the Premier *C. difficile* Toxin A test. The microorganism was cultured from 25% of specimens that were negative by both the cellular cytotoxicity assay and the Premier *C. difficile* Toxin A assay. For five of these, the ability to produce cytotoxin was determined, and four specimens were positive. No direct relationship between cytotoxin titer and the immunoassay reading could be established. In a multicenter study, the sensitivity and specificity of the Premier *C. difficile* Toxin A immunoassay and the cellular cytotoxicity assay were 86.6 and 99.0% and 93.9 and 99.8%, respectively (69). The positive and negative predictive values were 94.7 and 97.4% for the Premier *C. difficile* Toxin A immunoassay and 98.7 and 98.8% for the cellular cytotoxicity assay, respectively. Delmée et al. (70) have demonstrated a sensitivity and specificity of 88 and 95% for the Premier *C. difficile* Toxin A immunoassay.

In other studies, the VIDAS *C. difficile* Toxin A (CDA) (bioMérieux Vittek, Inc., Hazelwood, Mo.) enzyme immunoassay was evaluated (46, 147, 244, 289). For 88 specimens, the assay showed a sensitivity, specificity, and correlation of 92.3, 100, and 96.6%, respectively, when compared with the cellular cytotoxicity assay (46). The negative and positive predictive values have been shown to range from 97 to 99.7 and 85.7 to 94% respectively (244, 289). Although detection of *C. difficile* toxin by the VIDAS CDA enzyme immunoassay requires an expensive microplate reader, the system compares favorably with the cellular cytotoxicity assay and turnaround time was decreased (147, 244). In a study of 194 clinical specimens, Shanholtzer et al. (245) indicated that the usefulness of the VIDAS CDA enzyme immunoassay was diminished by a high percentage (19%) of results that could not be interpreted. Further, the VIDAS CDA immunoassay and Culturette Brand CDT latex agglutination tests were found to show 52 to 63 and 48 to 58% sensitivity, respectively, compared with 93 to 100 and 70 to 100% sensitivity, respectively, for culture and cytotoxin testing (245).

Several workers have evaluated the Bartels Enzyme Immunoassay (Baxter Healthcare Corp.) for the detection of *C. difficile* toxin A (77, 91, 103, 142, 198, 267, 289). Keiser et al. (142) found the assay to have a sensitivity and specificity of 94 and 96%, with positive and negative predictive values of 75 and 99%, respectively. Other studies have generally supported these results (198, 289). The assay uses toxin A-specific mouse immunoglobulin G bound to microwell strips. Addition of patient sample is followed by addition of rabbit immunoglobulins to *C. difficile* toxin A and peroxidase-labelled goat anti-rabbit antibodies.

Analytab Products (Plainview, N.Y.) has marketed the Analytab *C. difficile* A + B ELISA Test Kit (also marketed as the Cytoclone A + B EIA test) as a qualitative enzyme immunoassay for the detection of toxins A and B in feces from patients with *C. difficile*-associated disease. The test

has a high sensitivity and specificity (94.9 and 98.8%, respectively) and detects both toxins A and B of *C. difficile*. Although the reactivities of positive clinical samples may decrease with time because of toxin degradation, the test has few limitations. Doern et al. (72) evaluated the cellular cytotoxicity assay, Cytoclone A + B EIA test, and Premier *C. difficile* Toxin A enzyme immunoassay. The authors concluded that, in general, the cellular cytotoxicity assay is more sensitive to establish a laboratory diagnosis of *C. difficile*-associated gastrointestinal disease. Further testing is in progress to determine the possible use of this enzyme immunoassay system as a single assay for the detection of *C. difficile*-associated disease.

Several other investigators have developed enzyme immunoassays that detect toxin A and/or toxin B in stool specimens (7, 120, 149, 172, 280, 284, 296, 302). These tests are controversial, and their possible drawbacks include inability to detect, in most instances, both toxin A and toxin B of *C. difficile*, the lack of a commercial antiserum, misinterpretation of visually read results in clinical laboratories without a spectrophotometric plate reader, and the presence of substances in clinical samples that cause the antigen-antibody complex to dissociate from the microtiter assay plate.

#### Counterimmunoelectrophoresis

The detection of *C. difficile* toxin in fecal filtrates by counterimmunoelectrophoresis was used in some laboratories as an aid in the diagnosis of *C. difficile*-associated disease (239, 240, 285); however, numerous studies have not supported its use (151, 162, 241, 287). When compared with the cellular cytotoxicity assay, a false-negative rate ranging from 53 to 75% and a false-positive rate ranging from 10 to 24% were demonstrated. These high false-negative and false-positive rates appear to be related to purity of the antigen, specificity of the antibody, and insufficient level of the cytotoxin in clinical samples (133, 162, 218, 219, 241, 287, 297).

#### Fluorescent-Antibody Assays

Rabbit antiserum prepared against a viable isolate of *C. difficile* reacted strongly against 40 isolates of the microorganism but was also reactive against isolates of *C. sordellii*, *Clostridium bifermentans*, *Clostridium chauvoei*, and *Clostridium sporogenes* (293). Although results of the fluorescent-antibody test agreed with those of the cellular cytotoxicity assay and culture for 93% of the specimens, 62% of the fecal specimens from normal adults from whom *C. difficile* could not be cultured were positive. These results suggest that the use of fluorescent antibodies to detect *C. difficile* in clinical samples is not effective because of the lack of antibody specificity.

#### Polymerase Chain Reactions

Wren et al. (297) used the polymerase chain reaction to amplify a fragment of repeating sequence of the *C. difficile* toxin A gene. The reaction distinguished 58 toxic from 17 nontoxic strains. The toxin A probe was also positive for 2 strains of *C. sordellii*, known to be positive in the toxin A and B assays for *C. difficile*, but negative for 17 other *Clostridium* isolates. Kato et al. (139) have used the polymerase chain reaction to identify *C. difficile* isolates that produce toxin A. The assay reaction differentiated 35 toxic from 26 nontoxic isolates of *C. difficile* and showed a

negative response for 30 strains of 20 other *Clostridium* species and 2 toxic and 7 nontoxic strains of *C. sordellii*. The assay system was designed to amplify segments of nonrepeating and repeating sequences of the *C. difficile* toxin A gene (75). In conjunction with other studies (223, 297), polymerase chain reaction and Southern blot analyses with subgenomic probes also suggest that nontoxic *C. difficile* isolates lack the entire 8,133-bp toxin A gene.

Gumerlock et al. (114) developed a rapid, specific, and sensitive test for *C. difficile* by using polymerase chain reaction technology. A pair of primers that complemented specific segments of the *C. difficile* 16S rRNA gene and a nonradioactive biotinylated probe specific for *C. difficile* were investigated. The system allowed the detection of as few as 10 *C. difficile* microorganisms among a total of  $10^{10}$  to  $10^{11}$  bacterial cells per g of feces. It discriminated between *C. difficile* and related microorganisms, including *C. sordellii* and *C. bifermentans*, but was unable to differentiate between toxic and nontoxic *C. difficile* strains. It is believed that pathogenic strains of *C. difficile* are toxic, whereas nonpathogenic strains are nontoxic (157, 175).

Possible drawbacks of the polymerase chain reaction may include its inability to discriminate the human carrier state from overt disease and extended incubation time.

### CONCLUSION

*Clostridium difficile* is a common nosocomial pathogen that infects 15 to 25% of hospitalized patients. These patients often have no history of antibiotic treatment, and fecal specimens are positive when cultured for *C. difficile* or assessed with the latex agglutination test. In these instances, positive test results may reflect the asymptomatic carriage of *C. difficile* (189).

Cell culture to detect the presence of *C. difficile* does not assess toxin-producing capacity or delineate the carrier state. Further, the latex agglutination test does not detect toxin A or toxin B and appears to be nonspecific, often resulting in false-positive and false-negative reactions. These results, observed in symptomatic and asymptomatic patients, often lead to the indiscriminate treatment of individuals without disease or to the lack of treatment for individuals with a serious infection, thus subverting the ethical and legal obligations of the diagnostic laboratory. It is essential that laboratory tests be used in conjunction with a clinical history to establish a diagnosis of *C. difficile*-associated disease.

While the most appropriate laboratory test to use for the diagnosis of *C. difficile*-associated disease remains controversial, the cellular cytotoxicity assay with cultured mammalian cells is considered the "reference method" or "gold standard." The assay can detect a lower limit of approximately 1 pg of toxin B and 10 to 20 ng of toxin A when used with the appropriate antisera (171, 185). A positive cytotoxic response in the cellular cytotoxicity assay correlates well with the clinical signs and symptoms of disease. The assay can be completed with a 96-well microtiter plate containing cultured mammalian cells (74). If the clinical laboratory lacks a mammalian cell culture facility, prepared plates with cultured human foreskin cells can be acquired commercially, making complete cell culture facilities unnecessary.

Although controversial, enzyme immunoassays which detect both toxin A and toxin B appear to be useful for the diagnosis of *C. difficile*-associated disease. Other test systems, including the latex agglutination test, possess drawbacks that may result in unacceptable levels of false-negative

and false-positive reactions. Further studies are required to assess the importance of *C. difficile* clinical isolates which produce toxin B only or which do not appear to produce toxin A or toxin B.

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