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Diagnosis of *Clostridium difficile* Infection: an Ongoing Conundrum for Clinicians and for Clinical Laboratories

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SUMMARY

Clostridium difficile is a formidable nosocomial and community-acquired pathogen, causing clinical presentations ranging from asymptomatic colonization to self-limiting diarrhea to toxic megacolon and fulminant colitis. Since the early 2000s, the incidence of *C. difficile* disease has increased dramatically, and this is thought to be due to the emergence of new strain types. For many years, the mainstay of *C. difficile* disease diagnosis was enzyme immunoassays for detection of the *C. difficile* toxin(s), although it is now generally accepted that these assays lack sensitivity. A number of molecular assays are commercially available for the detection of *C. difficile*. This review covers the history and biology of *C.*

difficile and provides an in-depth discussion of the laboratory methods used for the diagnosis of *C. difficile* infection (CDI). In addition, strain typing methods for *C. difficile* and the evolving epidemiology of colonization and infection with this organism are discussed. Finally, considerations for diagnosing *C. difficile* disease in special patient populations, such as children, oncology patients, transplant patients, and patients with inflammatory bowel disease, are described. As detection of *C. difficile* in clinical specimens does not always equate with disease, the diagnosis of *C. difficile* infection continues to be a challenge for both laboratories and clinicians.

INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming, rod-shaped bacterium that exists in the soil and in the gastrointestinal tract of animals and humans. More than 100 strains exist based upon mutations in the genes on the pathogenicity locus (PaLoc) that encode various toxins (see "Genetics and Pathogenesis" below) (1, 2). There are two forms of the organism, a dormant spore form that is resistant to antibiotics and a vegetative form that can produce toxins and is susceptible to the activity of antibiotics. The organism and its toxigenic potential were discovered in 1935 by Hall and O'Toole, who noted it as a component of the normal fecal microbiota of newborn infants (3). Since it was difficult to cultivate *in vitro*, they named it *Bacillus difficilis* (3, 4). Later, investigators verified that *Bacillus difficilis* produced a toxin that was highly potent when injected intraperitoneally into guinea pigs (5).

Since the organism did not seem to be associated with human disease at that time, it was largely ignored until the antibiotic era. In 1974, Hafiz characterized the organism in more detail, including a description of glutamate dehydrogenase (GDH) (also known as common antigen), and showed that it was widely distributed in nature, including in the stools of many animals (4). Around that time, cases of pseudomembranous colitis (PMC), initially described as early as 1893 (6), became much more prevalent and were attributed primarily to *Staphylococcus aureus* (4). Oral vancomycin provided therapeutic success and became the treatment of choice for this disease (4). Also, in the early 1970s, Tedesco et al. noted that clindamycin treatment for anaerobic infections seemed to cause severe diarrhea in several patients. Those researchers performed a prospective study to assess the frequency of diarrhea associated with clindamycin treatment and to assess patients who developed diarrhea by colonoscopy. Remarkably, 42 of 200 patients developed diarrhea, and 20 patients had endoscopic evidence of PMC, which those researchers called "clindamycin colitis" (7). *S. aureus* was not recovered from the stool samples of these patients. The connection with *C. difficile* as the etiologic agent was not made at that time, nor was it made by Green et al., who studied cecitis induced by penicillin in animals; those investigators did not understand that the toxin that they recovered in tissue cultures in 1974 was produced by *C. difficile* (4).

A series of studies and observations by many groups followed the published observations in 1974. It was shown that oral vancomycin administration could prevent clindamycin colitis when administered simultaneously. Other studies showed that the potential agent of disease could be transferred from ill to healthy animals in cecal contents and that it likely produced a large protein toxin (4). Laughon et al. reported detection of a cytotoxic substance in the stool samples of patients with pseudomembra-

nous colitis (8). Te-Wen Chang and others characterized the morphological changes associated with antibiotic-associated colitis in animals and discovered that the cytopathic effects observed in fibroblast cells in tissue culture could be neutralized with *Clostridium sordellii* antitoxin, thus developing the cytotoxin assay as a potential diagnostic test method (9). In 1978, after multiple experiments, Bartlett et al. published the sentinel paper linking *C. difficile* toxin production to PMC in humans (10). Interestingly, several papers followed in which better diagnostic assays for *C. difficile* cytotoxin were sought, and enzyme immunoassays (EIAs) were developed. However, EIAs were shown to be about 10-fold less sensitive than the cytotoxin assay and were not pursued further by early investigators (4, 8).

During the 1980s and 1990s, *C. difficile* disease was not a major cause of mortality. However, in the new millennium, due to the "perfect storm" of epidemiological factors, host factors, and the emergence of organism traits that facilitate infection and disease, *C. difficile* infections (CDIs) have increased in frequency and severity. These important factors have resulted in reevaluation of diagnostic test methods and have stimulated the development of new assays.

THE ORGANISM—PATHOGENESIS AND DISEASE MANIFESTATIONS

Genetics and Pathogenesis

The emergence of "hypervirulent" *C. difficile* strains in the new millennium has sparked a renewed interest in creating better animal models of human disease and progress in phylogenetic studies to understand the pathophysiology of *C. difficile* infection. At least two strains have been completely sequenced—strain 630, an epidemic, restriction fragment length polymorphism (RFLP)-PCR toxinotype X clinical strain that is virulent and multidrug resistant, and a nontoxigenic strain, CD37 (ribotype 009) (11, 12). Several other isolates have had partial genomic characterization. A detailed discussion of the *C. difficile* genome is beyond the scope of this review, and the reader is referred to other references for information (11, 13). However, it is important to stress that such work has provided insights into how *C. difficile* survives in the environment and causes disease in humans and animals—information that may promote development of diagnostic tests and therapeutic interventions.

In order for *C. difficile* to cause disease, several important conditions must be met. A person must have contact with the spores of a toxin-producing strain of *C. difficile* in combination with alteration of the normal colonic microbiota, permitting colonization of the organism. Both animal models and studies of the human gut microbiome have elucidated the microbial and cellular interactions within this complex intestinal ecosystem (14). Also of importance is the host immune system, as evidenced by the higher rates of infection and worsening disease severity among the elderly and other persons who lack the ability to mount an effective humoral immune response (15, 16). Antitoxin antibodies may be protective and can also explain variations in disease presentations among immunocompetent hosts (15, 17).

The fact that antibiotics alter the gut microbiota has been established since the 1940s, shortly after streptomycin became available and investigators noted the impact of oral administration of this agent on the bacteria present in the feces of mice (14). In subsequent decades, these observations were pursued in animal models

of enteric diseases, including *C. difficile* infections, where it was discovered that animals who were pretreated with antibiotics prior to challenge with the enteric pathogen succumbed to infection compared to the untreated groups (18–20). In several models, the untreated animals often did not become colonized, or the endogenous microbiota outcompeted the challenge pathogen (18–20), a phenomenon referred to as colonization resistance (14).

Antibiotics are the major risk factor for the development of *C. difficile* disease because of the loss of endogenous microbiota that allows *C. difficile*, when present, to proliferate and invade. Several interesting studies of the gut microbiome using culture-independent methods have elucidated the significant alterations that follow antibiotic administration (21, 22). These studies have also shown that in patients recovering from *C. difficile* disease, recurrence may be related to a failure to restore the normally diverse microbial intestinal community (22, 23). The latter observation explains why fecal transplantation is so efficacious in the management of patients with recurrent *C. difficile* disease (24, 25). The precise protective mechanisms of the normal microbiota in preventing *C. difficile* disease are not completely elucidated. Several hypotheses that are being pursued include (i) the impact of gut microflora on bile acid transformation that impacts *C. difficile* spore germination; (ii) direct antagonism by the normal microbiota through production of bacteriocins, antimicrobials produced by these organisms that directly inhibit the growth of *C. difficile*; (iii) stimulation of innate immune responses by Toll-like receptor 5 signaling produced by intestinal microbiota; and (iv) competition between the microbiota and *C. difficile* for limited nutritional resources (14).

A significant portion of the *C. difficile* genome is involved in encoding factors that ensure survival in the gastrointestinal tract. These coding regions produce enzymes that assist with carbohydrate transport and metabolism, including *p*-hydroxyphenylacetate decarboxylase, which allows the organism to produce *p*-cresol, a bacteriostatic compound that may be inhibitory to intestinal microbes (11, 26). It is this compound that is responsible for the "horse barn" odor characteristic of *C. difficile*, a trait often used to identify the organism when grown in culture. In addition, *C. difficile* produces other compounds that allow it to utilize nitrogen and phospholipids found in the host's diet and to survive in the presence of bile acids (26).

A large segment (19.6 kb) of the genome found in toxigenic strains but lacking in nontoxigenic strains is the pathogenicity locus (PaLoc). The PaLoc contains five genes: *tcdA*, *tcdB*, *tcdC*, *tcdE*, and *tcdR* (1). *tcdA* and *tcdB* are in close proximity, separated by *tcdE*, and encode toxins A and B, respectively. The *tcdE* gene encodes a protein that is important for the release of toxins A and B from the cell (27). Upstream of *tcdB* is *tcdR*, which is a positive regulator of *tcdA* and *tcdB* expression (1, 27). Downstream of *tcdA* is *tcdC*, which functions as a negative regulator of toxin production during the exponential phase of growth (1, 28). We refer the reader to other articles with schematic representations of the PaLoc (26, 29–31). The other genes, *tcdA*, *tcdB*, *tcdE*, and *tcdR*, are expressed during stationary phase (1). Another important regulator of toxin gene expression is CodY, which is a global gene regulator that monitors environmental factors such as the presence of carbohydrates, amino acids, and other nutrients (32). CodY influences *tcdR* expression and, hence, toxin gene production and may be a potential target for future therapeutic agents (32).

In addition to toxins A and B, a third toxin, called binary toxin (see below), is also produced by some, but not all, strains of toxigenic *C. difficile*. The genes that encode binary toxin are not found in the PaLoc (26). Other important virulence factors that contribute to the pathogenesis of *C. difficile* include adhesins, fimbriae, flagella, a capsule, and a paracrystalline S-layer protein (important in cellular adhesion) (11, 26, 29, 33).

Roles of Toxin A, Toxin B, and Binary Toxin in Pathogenesis

Toxin A (TcdA) and toxin B (TcdB) are large clostridial toxins (205 kDa and 308 kDa, respectively). Similar to the toxins of other members of the clostridial toxin family, they cause disease through a variety of cytotoxic mechanisms, most notably the loss of the cytoskeletal structure, leading to cell rounding and cell death (1, 26, 29, 33). Originally, TcdA was believed to be more important in causing *C. difficile* disease. This thinking evolved based upon several observations in a variety of animal models. In both hamsters and mice, intragastric administration of TcdA, but not TcdB, resulted in intestinal fluid accumulation, diarrhea, hemorrhage, and death (29). Because the fluid accumulation in the rabbit ligated ileal loop model was akin to that seen with cholera toxin, TcdA was referred to as an enterotoxin (1, 29). Both TcdA and TcdB, when administered intraperitoneally, were observed to have similar potencies (29). TcdB is referred to as the cytotoxin because it is 100- to 1,000-fold more potent *in vitro* in cultured cells than TcdA (1). It has now been established that both toxins can cause significant disease, as evidenced by outbreaks of severe infection caused by TcdA-negative, TcdB-positive strains (34–36).

Both TcdA and TcdB are large, single-stranded, two-component proteins containing an enzymatically active A subunit and a B subunit that delivers the A subunit into the target cell. X-ray crystallography and small-angle X-ray scattering models have shown that each toxin has four structural domains (29, 37, 38). Production of active toxin involves four steps mediated by their respective domains. The C terminus of both toxins contains the receptor binding domain. This domain has short combined repetitive oligopeptides (CROPs) that, in the case of TcdA, bind to saccharides in the human glycoprotein receptor gp96; the receptor for toxin B binding is unknown (29, 38).

The toxins enter cells by clathrin-mediated endocytosis, where they must escape from the endosome so that glucosyltransferase cleavage and activity may proceed (1, 29). This is achieved by pore formation in the endosomal membrane mediated by hydrophobic segments of the central delivery domain after the region has been structurally changed by endosomal acidification (1, 29). Once in the cytosol, the A component of each toxin is cleaved at a cysteine protease domain adjacent to the glucosyltransferase domain. This cleavage is mediated by host cell inositol hexakisphosphate (InsP6) (26, 29, 39), resulting in release of biologically active toxin, a 63-kDa glucosyltransferase that resides at the N termini of both toxins.

As mentioned above, active toxin inactivates small Rho and other GTPases, affecting their interactions with regulatory molecules and interrupting vital signaling pathways (29). Cells round up, shrink, and die, leading to significant loss of the intestinal epithelial barrier, and tight junctions are disrupted, permitting neutrophil migration. In addition, both toxins stimulate the release of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-8 from activated macrophages. This subsequently leads to neutrophil recruitment, inciting an inflammatory response (40); neutrophil ac-

cumulation is responsible for the pseudomembrane formation seen in severe colitis.

Approximately 6 to 12.5% of strains of *C. difficile* produce another toxin, called *C. difficile* transferase (CDT), also known as binary toxin, which is encoded by the Cdt locus (CdtLoc) (26). Binary toxin is so called because it is composed of two subunits, namely, CDTa and CDTb, that are produced and secreted from the cell as two separate polypeptides (41). These polypeptides combine into a potent cytotoxin, CDT.

CDT is an ADP-ribosyltransferase that disrupts the cytoskeleton of the cell, leading to cell rounding, loss of fluids, and eventually cell death (40, 41). Like TcdA and TcdB, CDT is a classical AB toxin. The target cell receptors necessary for the binding subunit (CDTb) are unknown. Toxicity of CDT involves cell entry via endocytosis, pore formation that delivers active toxin into the cytosol, and ADP-ribosylation of G-actin (40, 41). The precise contribution of CDT to human disease is still being elucidated. In animal models, CDT-positive (CDT$^+$) strains that lack TcdA and TcdB do not seem to cause disease, although massive edema was seen in rabbit ileal loops (42). More recent findings indicate that CDT may play a role in intestinal colonization through induction of microtubule formation on epithelial cell surfaces, leading to enhanced adherence (43). Since CDT production appears to be epidemiologically associated with strains producing higher fatality rates (30, 40, 44), this observation and the findings from animal studies have led to the hypothesis that CDT contributes to an increased severity of disease (40).

Multilocus sequencing typing (MLST) and whole-genome sequencing (WGS) of the PaLoc reveal that *C. difficile* can be divided into five genetic groups or clades (designated clades 1 to 5) that continue to evolve at the strain level (44, 45). Within these clades, strains that appear to be associated with more severe disease and increased mortality have emerged—the so called "hypervirulent" types. Examples of these strains include ribotype 027 (toxinotype III, ST-1, BI/NAP1) and ribotype 078 (ST-11) strains. Outbreaks related to these strains increased dramatically at the beginning of the new millennium and continue to cause about 50% of cases in some geographic locations (30, 34). More than a decade of research has determined that there are several factors contributing to the successful dissemination and enhanced pathogenicity of these clones. These factors include (i) fluoroquinolone resistance (30), (ii) higher sporulation rates (46), (iii) rapid internalization and pore formation of TcdB due to divergence in the receptor binding domain (29, 47, 48), (iv) a 20-fold increase in toxin production related to mutations in *tcdC* (49, 50), (v) enhanced cytotoxicity (47), and (vi) production of binary toxin. These and other studies will hopefully further our understanding of the molecular epidemiology of *C. difficile*.

Clinical Manifestations

C. difficile infection can run the gamut from asymptomatic colonization to severe PMC, toxic megacolon, and death. The type of disease and severity of disease are related to the organism factors described above and patient risk factors, including the presence of neutralizing antibody against TcdA and TcdB (16). Patients with clinical symptoms can be stratified into mild-to-moderate illness, severe illness, and fulminant disease (51, 52). This stratification is important in determining the need for treatment, the type of antimicrobial agent that may be required for treatment, whether surgery is needed to control disease, and the need for supportive care such as intensive care unit admission (52). Watery, non-bloody diarrhea, defined as 3 or more stools per 24-h period, is the hallmark of symptomatic illness (51, 52). Mild disease is characterized by diarrhea in the absence of signs and symptoms of colitis. Patients with moderate disease have diarrhea with evidence of colitis characterized by fever and abdominal cramps, usually in the lower quadrants. Laboratory abnormalities in mild and moderate disease include a leukocytosis level of 15,000 cells/μl or lower (51, 52) and a serum creatinine level less than 1.5 times the premorbid level (52). The Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA) guidelines define severe disease as colitis associated with a leukocyte count that is 15,000 cells/μl or higher and a serum creatinine level equal to or greater than 1.5 times the premorbid level (52). Other characteristics of severe disease include markedly elevated temperature reaching 40°C, PMC, and hypoalbuminemia (serum albumin level of <2.5 mg/dl) (51, 52). Fulminant *C. difficile* occurs in <5% of patients and is characterized by severe abdominal pain, profuse diarrhea, or sometimes no diarrhea, as the patient rapidly progresses to development of an ileus or toxic megacolon, a condition in which the colon becomes distended greater than 6 cm and is in danger of perforation (51, 53). This is an ominous development and may indicate the need for emergent surgery. The leukocyte count may be as high as 50,000 cells/μl or higher, and a serum lactate level of >5 mmol/liter portends a poor outcome (53). Several factors have been associated with increased mortality, the most frequent of which is an age of >70 years. Comorbid conditions that contribute to the risk of dying within 30 days include cognitive impairment and liver, renal, and ischemic heart diseases (54).

LABORATORY METHODS FOR *CLOSTRIDIUM DIFFICILE* DETECTION

Methods

Clostridium difficile infection is a clinical diagnosis supported by laboratory findings. The laboratory methods used historically for *C. difficile* detection had prolonged turnaround times, which limited their practicality for diagnostic testing. As a result of this, when enzyme immunoassays (EIAs) emerged for *C. difficile* detection, they were widely adopted by many laboratories because of the speed, convenience, and economy of using these methods. However, it was ultimately demonstrated that these methods lacked analytical sensitivity, which ushered in an era of nucleic acid-based techniques for detection of *C. difficile* in clinical specimens. The next section provides an overview of the laboratory methods used for the diagnosis of *C. difficile* disease.

Cell culture cytotoxicity neutralization assay. The cell culture cytotoxicity neutralization assay (CCCNA) is performed by preparation of a stool filtrate, which is then applied onto a monolayer of an appropriate cell line. A number of different cell lines have been used for this purpose, such as human foreskin fibroblasts, human diploid fibroblasts, Vero cells, McCoy cells, MRC-5 lung fibroblasts, and Hep2 cells. Following 24 to 48 h of incubation, cells are observed for toxin-induced cytopathic effect (CPE); if CPE is observed, a neutralization assay is performed to ensure that the CPE is attributable to *C. difficile* toxins rather than nonspecific toxicity. The neutralization assay is performed by using either *C. sordellii* or *C. difficile* antiserum. Although toxin B is primarily detected in this assay, toxin A is also detected to some extent (55).

CCCNA has historically been considered the gold standard

(56); however, this method is now considered by most experts to lack the desired sensitivity to be the gold standard for assay comparison studies. The reported sensitivities for CCCNAs are quite variable, ranging from 65 to 90% (52, 57–61). Compared to toxigenic culture, CCCNA has a sensitivity of approximately 75 to 85% (56), and the level of agreement between the two assays can vary considerably between test sites. The performance characteristics of the CCCNA vary depending on a number of factors. The toxin can degrade in the specimen, so if there is a delay in transport, false-negative results can occur. In addition, results can vary depending on the cell line used, the timing between symptom onset and performance of the assay, and pretreatment of the patient with antimicrobial therapy active against *C. difficile*; analytical factors, such as the method of preparation of the fecal filtrate, can also impact the sensitivity of this assay (55, 57, 60, 62–65). CCCNA has fallen out of favor as a routine diagnostic test as a result of inadequate sensitivity, the relatively prolonged turnaround time (24 to 48 h), and the requirement for expertise in maintenance of cell cultures and interpretation of results.

Toxigenic culture. Toxigenic culture for *C. difficile* is based upon isolating the organism from fecal specimens and determining if the recovered isolate is a toxin-producing strain. There have been many different methods reported for this purpose. The essence of all of the methods is based on using anaerobic agar or broth culture with selective and differential agents to inhibit overgrowth of other fecal flora while enhancing the recovery of *C. difficile*. To date, there is no consensus on the best method for recovery of the organism in culture. Some investigators have employed a heat shock or alcohol shock prior to inoculation to enhance spores of *C. difficile* and inhibit growth of other organisms (66–68). A variety of different medium formulations have been described, many of which take advantage of the fact that *C. difficile* can ferment fructose (69, 70). The medium type most commonly used is cycloserine, cefoxitin, and fructose agar (CCFA) (71), but a number of variants on the theme of CCFA have been reported, such as cefoxitin cycloserine egg yolk agar (CCEY), also known as Brazier's medium, and cycloserine cefoxitin egg yolk agar with lysozyme (CCEYL) (72). A variety of agents have been added to these different medium formulations to enhance vegetation of spores, such as lysozyme and taurocholate (73, 74). Broth enrichment prior to plating onto solid agar appears to enhance recovery (67, 74). Regardless of the medium or method used, it is essential that the medium is prereduced prior to inoculation of the specimen to maximize recovery of the organism. Cultures are typically incubated for a minimum of 48 h and are frequently held for up to 7 days before being reported as negative.

Few studies have simultaneously compared different media and culture conditions for recovery of *C. difficile* from samples. A recent systematic evaluation compared different medium types, preincubation steps, and direct plating onto solid medium versus the use of broth enrichment culture (67, 74, 75). Hink and colleagues observed that the most sensitive method for *C. difficile* isolation from both stool and swab specimens was heat shock at 80°C prior to inoculation of the specimen into cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (67).

Once colonies suspicious for *C. difficile* are isolated in culture, the organism is typically identified based on Gram stain, colony morphology, "horse barn" odor, and biochemical testing, such as detection of hydrolysis of L-proline-naphthylamide ("PRO Disk" positive) and spot indole. *C. difficile* isolates will exhibit yellow-

green fluorescence under UV light (72). Commercial biochemical methods for identification, such as the RapidANA (Remel, Lenexa, KS) test, may also be used, and identification using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is used by some laboratories.

When *C. difficile* has been identified, isolates must be tested for the ability to produce toxins. Frequently, this is performed by growing the *C. difficile* isolate in broth (such as prereduced brain heart infusion or chopped meat broth) and subsequently performing CCCNA on the culture supernatant. Alternatively, the supernatant can be tested for toxin production by using commercially available toxin EIAs; this is a rapid and reliable method for toxin detection from cultured isolates when the EIA is positive (76, 77). She et al. reported the Meridian Premier Toxins A & B and the TechLab Tox A/B II assays to be 87.1% and 89.2% sensitive, respectively, for the detection of toxin-producing strains compared to CCCNA performed by using human foreskin fibroblast cells (76).

The fecal specimen for culture may be a stool specimen, rectal swab, or perirectal swab; a recent study suggested that perirectal swab specimens are almost as sensitive as "cup" stool specimens for detection of *C. difficile* in symptomatic patients for whom diagnostic testing for *C. difficile* is sought (95.7% sensitive compared to stool samples) (78). Even for patients with diarrhea, at times it can be difficult to obtain a stool specimen due to a variety of factors, such as the mental status of the patient and the availability of a collection container at the time of defecation.

In addition to conventional culture methods, chromogenic media have been developed for *C. difficile*; these media can expedite recovery of *C. difficile* from fecal specimens. For example, presumptive identification of *C. difficile* can be made based on black colonies on bioMérieux chromID agar. A recent report compared bioMérieux chromID *C. difficile* agar to taurocholate cycloserine cefoxitin agar and bioMérieux CLO medium (79). Four hundred six consecutive diarrheal stool samples from hospitalized patients were cultured; the gold standard for comparison was the recovery of *C. difficile* in the sample by using any of the culture methods. chromID agar was 74% sensitive at 24 h and 87% sensitive at 48 h (79). In a similar study, Perry et al. reported on a prototype of a chromogenic medium for recovery of *C. difficile* from fecal specimens (80); on this medium, *C. difficile* appears as black colonies on a clear background, similar to chromID agar. This prototype agar was compared to bioMérieux CLO medium, BBL *Clostridium difficile* agar, Oxoid *Clostridium difficile* agar with defibrinated horse blood and moxalactam-norfloxacin supplement, and CCEY with and without supplementation with lysozyme. In total, 226 toxin-positive and 113 toxin-negative stool specimens were evaluated under the 5 different culture conditions (toxin status of stool specimens was determined by using the Vidas immunoassay). The sensitivities of the prototype chromogenic agar were 97% and 99% at 24 and 48 h of incubation, respectively (80). The recovery of *C. difficile* was significantly better with the prototype medium than with all other medium types evaluated in this study.

The chromID *C. difficile* medium has also been evaluated for cultivation of *C. difficile* from environmental surfaces within a hospital setting and compared to cefoxitin cycloserine egg yolk agar with lysozyme for this purpose (81). By using a combination of both media, *C. difficile* was recovered from 21% of the sites sampled, but the sensitivity of chromID agar was significantly

TABLE 1 Examples of studies evaluating the performance characteristics of EIAs for detection of *C. difficile* toxins[a]

Assay	Format	Gold standard	No. of samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
Meridian Premier Toxins A & B	Microwell EIA	CCCNA	540	96.8	94.3	50.9	99.8	88
Meridian Premier Toxins A & B	Microwell EIA	CCCNA	446	98.7	97.3	ND	ND	94
Meridian Premier Toxins A & B	Microwell EIA	Toxigenic culture	200	48	98	88	87	106
Meridian Premier Toxins A & B	Microwell EIA	Detection of *tcdB* by ≥2 molecular methods	81	42.3	100	100	78.6	90
Meridian Premier Toxins A & B	Microwell EIA	Toxigenic culture	432	58.3	94.7	68.9	87.0	87
Meridian Premier Toxins A & B	Microwell EIA	Toxigenic culture	596	80.8	97.5	78.0	97.9	58
		CCNA		91.7	97.1	78.0	99.1	
TechLab Tox A/B Quik Chek	Lateral flow EIA	Aggregate of EIAs and toxigenic culture	284	59.6	99.2	Not given	Not given	91
TechLab Tox A/B Quik Check	Lateral flow EIA	Toxigenic culture	596	74.4	98.9	88.7	97.2	58
		CCNA		84.3	98.6	86.8	98.3	
ImmunoCard Toxin A & B	Lateral flow EIA	Toxigenic culture	150	47	99.2	88.9	93.6	61
Immunocard Toxin A & B	Lateral flow EIA	Toxigenic culture	200	48	99	91	87	106
Remel Xpect *C. difficile* A/B	Lateral flow EIA	Toxigenic culture	200	48	84	88	87	106
Remel Xpect *C. difficile* A/B	Lateral flow EIA	Toxigenic culture	596	68.8	99.4	92.4	96.6	58
		CCNA		77.8	98.8	87.5	97.6	
Remel ProSpecT	Microwell EIA	Toxigenic culture	596	81.6	93.3	57.4	97.9	58
		CCNA		89.8	92.6	57.5	98.8	
Wampole Tox A/B Quik Chek	Lateral flow EIA	Toxigenic culture	360	43.18	99.68			89
Vidas *C. difficile* Toxin A/B	Solid-phase, enzyme-linked fluorescent assay	Toxigenic culture	150	58.8	89.4	62.5	96.7	61
Vidas *C. difficile* Toxin A/B	Solid-phase, enzyme-linked fluorescent assay	Toxigenic culture	596	80.0	97.3	76.4	97.8	58
		CCCNA		89.8	96.7	75.3	98.8	

[a] CCCNA, cell culture cytotoxicity neutralization assay; ND, not determined.

higher than that of CCEY with lysozyme (87.6% compared to 26.6%) (81).

Chromogenic media are usually more expensive than other medium types; this is typically attributed to the proprietary and chromogenic substrates in the media. However, the studies to date suggest that chromogenic media are sensitive for recovery of *C. difficile* and may speed the time to presumptive identification of isolates.

Toxigenic culture is typically considered to be a reference method rather than a diagnostic method. It is imperative that at least a subset of laboratories maintain expertise in *C. difficile* culture for method comparison and epidemiological studies, but the labor requirement and turnaround time are not usually considered practical for routine diagnostic use. In addition, toxigenic culture detects the ability of a *C. difficile* strain to produce toxin *in vitro* and does not necessarily inform *in vivo* production of toxin in the host. In summary, toxigenic culture is now considered by many to be the gold standard for *C. difficile* detection in fecal specimens (82, 83). The SHEA/IDSA guidelines support the use of toxigenic culture as the gold standard in method comparison studies (52). However, others argue that although toxigenic culture may result in more positive specimens, it might not be a superior test for the diagnosis of clinically actionable disease compared to CCCNA (56).

Toxin immunoassays. Enzyme immunoassays for *C. difficile* diagnostics use monoclonal or polyclonal antibodies directed against *C. difficile* toxins. Until very recently, EIAs for toxin detection have been the most frequently used assays in clinical laboratories for *C. difficile* detection. There are a number of commercially available EIAs for *C. difficile* toxins, including rapid immuno-

chromatographic/lateral flow membrane immunoassays and microwell and solid-phase assays. It should be noted that although some of the early assays detected toxin A exclusively, it is now generally recommended that assays detect both toxin A and toxin B, as toxin A-negative disease-causing strains have been well documented (35, 84–86) (also described above). Overall, the cost per test for these assays is low. The hands-on time required to perform the test varies depending upon the test type. The sensitivity and specificity that have been reported for these assays vary widely, from approximately 40% to 100% (58, 61, 87–94). A summary of several of the studies evaluating different EIAs for detection of *C. difficile* toxins can be found in Table 1. The gold standard used to evaluate these assays is variable and is either CCCNA or toxigenic culture, as indicated in Table 1. A study by Eastwood et al. evaluated a number of EIAs by using both gold standards and highlighted the difference in sensitivity and specificity reported for comparator assays depending upon the gold standard used (58).

Although the poor sensitivity of toxin EIAs for *C. difficile* is well established, the clinical significance of this has not been well documented. In an attempt to address this question, in a retrospective study, Polage and colleagues reviewed the charts of patients at the University of California—Davis Medical Center who had a *C. difficile* toxin test ordered between 1 January 2005 and 21 December 2009 (95). This cohort included 925 toxin-positive patients and 6,121 toxin-negative patients (95). Upon chart review, only 1 out of the 6,121 toxin-negative patients exhibited PMC, and there were no documented cases of complications that could be attributed to *C. difficile* disease in any of these patients, such as toxic megacolon, fulminant colitis, or colectomy. A small proportion (5.3%) of the toxin-negative patients received empirical treatment

TABLE 2 Examples of results of investigations evaluating the performance characteristics of assays for GDH detection[a]

Assay	Format	Gold standard	No. of samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
C. Diff Quik Chek	Lateral flow EIA	Detection of *tcdB* by ≥2 molecular methods	81	96.2	76.4	Not given	Not given	90
C. Diff Quik Chek	Lateral flow EIA	Toxigenic culture	401	93.5	96.9	87.9	98.4	110
C. Diff Chek-60	Microwell EIA	Aggregate of EIAs and toxigenic culture	284	100	94.2	Not given	Not given	91
C. Diff Chek-60	Microwell EIA	Toxigenic culture CCCNA	564	87.6 / 90.1	94.3 / 92.9	78 / 71.3	97.1 / 98.0	58
C. Diff Chek-60	Microwell EIA	Toxigenic culture	497	93.5	98	91.6	98.5	116
C. Diff Chek-60	Microwell EIA	Toxigenic culture and PCR	1,468	93.4	96.6	75.9	99.2	113
C. Diff Complete (GDH component)	Lateral flow EIA	Toxigenic culture	60	100	93.3	93.8	100	115

[a] By definition, GDH assays are screening assays and are not for definitive reporting of *C. difficile*. In this table, specificity is reported for GDH assays performed without confirmatory testing.

for *C. difficile* infection, regardless of the toxin result. This study suggested that the clinical impact of the reduced sensitivity of toxin EIAs for *C. difficile* was minimal, but more studies are needed to fully evaluate this issue.

Although the specificity of the toxin EIAs is generally superior to the sensitivity of this method, specificities vary, and some assays possess inadequate positive predictive values (PPVs) for a diagnostic test (58, 61, 96, 97). An abundance of false-positive results can be just as problematic as false-negative results. This phenomenon was highlighted in a report by Han et al., who observed that a change in the toxin EIA brand used for routine testing (from ProSpecT Toxin A/B [Remel] to *C. difficile* Tox A/B II [TechLab]) resulted in an apparent decrease in *C. difficile* infection from 23.52 to 8.69 cases per 10,000 patient days (97). Using the ProSpecT test, 19.7% of patients were positive with the first test, compared to 9.1% of patients positive with the first test using the TechLab Tox A/B II assay (97). A reduction in anti-CDI therapy followed as a result of fewer positive tests, but no increase in mortality occurred, suggesting that the decreased incidence was a result of improved assay specificity of the toxin A/B assay (97).

There are a number of reasons for the heterogeneity in performance characteristics of these assays, including, but not limited to, geography, circulating strain types (and, therefore, potentially differences in the antigenic features of the toxins that are expressed), the gold standard used for assessment of the assay, host antibody binding to toxin in the gastrointestinal tract, interlaboratory technical variance, institutional variance in ordering practices for *C. difficile* testing and/or specimen transport time, and whether fresh or frozen/stored stool samples are analyzed. To muddy the waters even further, in some studies, investigators have modified manufacturers' recommendations for the assays, such as the optical density (OD) to be considered "positive" for the microwell format EIAs (98). In addition, clinical signs and symptoms have only rarely been taken into consideration when evaluating the performance characteristics of these toxin EIAs (61, 95, 96, 99). Since the lack of sensitivity of *C. difficile* EIAs is a well-known problem within the medical community, common practice has been to order "*C. difficile* testing times three." This approach only compounds the problem; with the poor specificity of these assays, the number of false positives exceeds the number of true positives with each round of testing, especially in a low-prevalence setting.

Due to the inadequate sensitivity of toxin EIAs for *C. difficile* testing, this method is not considered the best way to make a diagnosis of *C. difficile* infection (52, 58, 100, 101). The SHEA/IDSA guidance document outlines that EIAs should no longer be considered adequate stand-alone tests for the diagnosis of *C. difficile* infection (52).

Glutamate dehydrogenase. Glutamate dehydrogenase (GDH) is a metabolic enzyme encoded by *gluD*. This antigen is produced at high levels in all isolates of *C. difficile*, including both toxigenic and nontoxigenic strains. In addition, GDH of *C. difficile* is known to cross-react with that of *C. sordellii*. Therefore, GDH represents a screening test for *C. difficile* disease, and positive assays must be followed up with a confirmatory test, such as a toxin EIA or a molecular test for detection of toxin genes. Overall, the studies performed to date suggest that GDH exhibits high sensitivity as a screening test for *C. difficile* infection and exhibits a favorable negative predictive value (NPV). Some of these studies are summarized in Table 2 (58, 65, 91, 102–116).

Similar to toxin EIAs, GDH assays are available in microwell EIA and lateral flow immunochromatographic formats. The sensitivity (80 to 100%) and NPV for GDH assays appear to be similar regardless of the specific GDH assay type used or the gold standard used to evaluate the assay (108, 113, 116, 117). GDH testing can efficiently provide a rapid turnaround time for negative results, with minimal hands-on time. The cost per test is low, especially compared to the cost of molecular methods.

GDH can be an attractive screening assay to quickly rule out *C. difficile* disease for laboratories that do not have access to or expertise in performance of molecular testing. However, some controversy exists regarding the adequacy of GDH assays to rule out *C. difficile* disease. In order to be a reliable and effective screening method, GDH should be highly conserved among *C. difficile* strains. A report by Tenover et al. raised concern that the sensitivity of GDH assays may vary by strain type; namely, reduced sensitivity for detection of strains of ribotypes 002, 007, and 106 was observed in this study (118). Carman et al. evaluated 104 isolates of *C. difficile*, corresponding to 77 different ribotypes (including both toxigenic and nontoxigenic strains, with ribotypes in common with those in the study by Tenover et al.), from different areas around the world (119). Most of the isolates had been recovered from 2004 to 2011. All of the strains of the ribotypes evaluated carried *gluD*, and predicted amino acid sequences of almost

TABLE 3 Performance characteristics of EIAs combining GDH detection and toxin EIA for *C. difficile* detection

Assay	Format	Gold standard	No. of samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
TechLab C. Diff Quik Check Complete	Lateral flow EIA	Detection of *tcdB* by ≥2 molecular methods	81	61.5	100	100	84.6	90
TechLab C. Diff Quik Chek Complete	Lateral flow EIA	Toxigenic culture	200 (all from pediatric patients)	70.8	97.4	89.5	91.4	122
TechLab C. Diff Quik Chek Complete	Lateral flow EIA	Aggregate of EIA and toxigenic culture	284	60.0	99.6	Not given	Not given	91
C. Diff Quik Chek plus Meridian Premier Toxins A & B	Lateral flow EIA/ microwell EIA	Detection of *tcdB* by ≥2 molecular methods	81	42.3	100	100	78.6	90
C. Diff Chek-60 with Meridian Premier Toxins A & B	Both are microwell EIAs	Toxigenic culture	432	55.6	98.3	87	91.7	87

all of the isolates were identical. All of the isolates produced GDH that was readily detected by using a number of commercial assays, including C. Diff Chek-60, C. Diff Quik Chek, and C. Diff Quik Chek Complete (119). These results suggest that the performance characteristics of commercial GDH assays are not dependent upon the *C. difficile* ribotype.

Goldenberg et al. evaluated the performance of the C. Diff Chek-60 GDH assay by using 64 isolates of *C. difficile* that could be grouped into six ribotypes: ribotypes 002, 005, 023, 027, 078, and 106 (120). Although this study used cultured isolates rather than direct testing of stool samples, there was no significant difference in the performance of the GDH assay for any of the ribotypes studied. Even when the cultures were diluted 1:100, the OD value for the GDH EIA did not vary significantly between any of the ribotypes tested (120).

Based on the studies Carman et al. and Goldenberg et al., it seems unlikely that regional or geographic differences in strain type account for the variance reported for GDH assays, although this cannot be completely excluded (118–121). For method comparison studies, it is not uncommon for the testing to be performed on frozen stool specimens; it is unknown if the freeze-thaw could impact assay results. This could be a confounding factor to consider when evaluating the variance between GDH assay performances in different method comparison studies.

Combination testing—GDH detection and toxin EIA. Some EIAs that combine GDH detection and a toxin EIA in one test have recently come on the market. These combination assays are relatively rapid, and the cost per test is less than that of the molecular methods. The GDH component of these assays appears to have a sensitivity similar to those of stand-alone GDH assays, but the toxin EIA component appears to suffer from the same sensitivity issues as conventional toxin EIAs (87, 90, 91, 122). Some of the studies evaluating these combination assays are described in Table 3. In general, samples that are GDH and toxin negative by these assays can be reported as negative with relatively high confidence, and GDH- and toxin-positive samples can be reported as positive. Samples that are GDH positive but toxin negative should have confirmatory testing performed (such as CCCNA or a molecular assay) to rule out *C. difficile* disease (100, 123, 124).

Molecular platforms for direct detection of *Clostridium difficile* in clinical specimens. The use of nucleic acid amplification methods for the detection of *C. difficile* in fecal samples began to appear in the literature in the early 1990s (125–127). These early assays used conventional PCR methods and targeted a variety of genes, including the *tcdA*, *tcdB*, and 16S rRNA genes. Extractions were cumbersome and time-consuming, and PCR products were de-

tected by gel electrophoresis and/or Southern blot analysis (126, 127). An additional observed pitfall with some of the early primer designs was cross-reactivity with other *Clostridium* species (125). However, sensitivity was found to be significantly better than those of existing anaerobic culture methods (127) and CCCNAs (128).

A decade later, methods of DNA extraction from fecal samples have improved because of the availability of kits that use a mixture of RNases, proteases, and other proprietary reagents in combination with spin columns for nucleic acid concentration and purification to replace cumbersome phenol-chloroform extraction procedures. Nucleic acid amplification techniques (NAATs) have improved, and real-time PCR instruments such as the Cepheid SmartCycler (Sunnyvale, CA), the Roche LightCycler (Pleasanton, CA), and the iCycler IQ (Bio-Rad Inc., Hercules, CA) became available, making laboratory-developed assays easier to verify and implement (59, 88, 129). Several laboratories have reported successful verification and implementation of laboratory-developed quantitative real-time PCR (qPCR) assays for clinical use before the availability of a U.S. Food and Drug Administration (FDA)-cleared platform (59, 106).

Despite these advances, it was 2009 before the first nucleic acid amplification assay for *C. difficile* detection in stool samples, the BD GeneOhm Cdiff assay (BD Diagnostics Inc., Sparks, MD), was approved by the FDA. The BD GeneOhm qPCR assay amplifies conserved regions of *tcdB* and detects the amplified products by using fluorogenic target-specific hybridization probes (molecular beacons) (60). Amplification, detection, and interpretation of the qualitative results are performed by using the Cepheid Smart-Cycler (Table 4). A built-in internal control alerts the user to problems with inhibition. The current FDA-cleared platform has a manual DNA extraction step and several pipetting steps before the vials are loaded onto the instrument. The total assay time ranges from 75 to 90 min depending upon the number of samples. An automated version, BD Max Cdiff, has recently received FDA clearance in the United States (89) and significantly reduces the setup time. The assay involves the use of a 10-μl inoculating loop to transfer liquid or unformed stool to the proprietary sample buffer. The sample buffer tube is placed into the BD Max instrument along with a reagent strip that contains all of the reagents necessary for sample processing and qPCR. The instrument moves a sample from one tube to the next on the reagent strip. Like the manual BD GeneOhm assay, the BD Max Cdiff assay amplifies *tcdB*. However, achromopeptidase is used for bacterial lysis in the newer version of the test (89). Amplified targets are detected with hybridization probes labeled with quenched fluorophores. A disposable microfluidic cartridge is where the amplifi-

TABLE 4 Available FDA-cleared molecular assays for detection of *Clostridium difficile* in fecal samples[a]

Assay	Target(s)	Chemistry(ies)	Extraction type	Unresolved/invalid rate (%)	TAT (min)
BD GeneOhm	*tcdB*	qPCR/molecular beacons	Manual	0.5–7.3	75–90
BD Max		Achromopeptidase lysis; qPCR/molecular beacons	Automated		120
ProDesse ProGastro Cd	*tcdB*	qPCR	Automated (Easy Mag)	2.7	180
Cepheid GeneXpert	*tcdB*, *cdtA*, *tcdC* nt 117 deletion	Multiplex qPCR	Automated (Infinity)	NA	29–45
Meridian Illumigene	*tcdA*	Loop-mediated isothermal amplification	Manual Illumipro-10 reader	0.8–4.4	70
Focus Technologies Simplexa	*tcdB*	qPCR, bifunctional fluorescent probe-primers	None	NA	60
Great Basin Portrait Analyzer	*tcdB*	Helicase-dependent amplification; microarray detection		NA	70
Quidel AmpliVue *C. difficile* Assay	*tcdA*	Helicase-dependent amplification; visual evaluation of results using handheld cassette	Manual	0.5	80
Nanosphere Verigene	*tcdA*, *tcdB*, *cdt* nt 117 deletion	PCR combined with gold particle probe capture and silver signal amplification on an array	Automated	2.4	150

[a] *tcdA*, toxin A gene; *tcdB*, toxin B gene; *cdt*, binary toxin gene; nt, nucleotide; NA, not available; TAT, turnaround time.

cation, detection, and interpretation of the signals take place. This cartridge is sealed to prevent contamination. The assay contains an internal sample processing control, which is read on an optical channel of the instrument separate from the patient samples. The overall time to completion is about 3 h, depending upon the total number of samples.

The performance characteristics of the BD GeneOhm Cdiff assay and the BD Max Cdiff assay along with those of the other FDA-cleared assays are summarized in Table 5 (57, 60, 89, 96, 107, 122, 130–134). Only those published studies which used a recognized reference method, such as toxigenic culture or a cell culture cytotoxicity neutralization assay, on all of the samples tested are included in Table 5. In cases where no published papers are available, data have been summarized from the manufacturer's product package insert. It is important to note that very few papers include clinical information, specifically with respect to whether patients met recognized case definitions as specified by clinical practice guidelines (52).

The ProDesse ProGastro Cd assay (Hologic Gen-Probe Inc., San Diego, CA) detects *tcdB* in fecal samples. A portion of the stool sample is diluted in stool transport and recovery (STAR) buffer, followed by centrifugation to remove solid particles (stool clarifi-

cation). The internal control is added to the clarified stool sample prior to extraction. Nucleic acid extraction and purification occur on the NucliSENS easyMag instrument (bioMérieux, Durham, NC). Amplification and detection using TaqMan chemistry, as well as interpretation of results, which are reported qualitatively, are performed with the SmartCyclerII instrument (Cepheid Inc., Sunnyvale, CA). Of all of the commercially available NAATs, this assay takes the longest to perform (Table 4). Performance characteristics are summarized in Table 5 (130).

Detection of not only *tcdB* but also the binary toxin genes and the deletion at nucleotide 117 on *tcdC* (Δ117) as surrogate markers for presumptive identification of 027/NAP1/BI strains are unique features of the Xpert *C. difficile* Epi assay (Cepheid Inc., Sunnyvale, CA). Users may also buy a version that detects only *tcdB*. This assay is among the simplest to perform and is also the most rapid of the available NAATs. A scored swab containing fecal material is inserted and broken off into an elution vial. After a vortex step, the liquid from the vial is dispensed into the sample port of the test cartridge that contains all of the reagents for amplification and detection as well as the internal control. The cartridge is subsequently loaded into the GeneXpert instrument. The company has a larger and more automated instrument, called the

TABLE 5 Published performance characteristics of currently available NAATs[c]

Assay	Sensitivity (%) (range)	Specificity (%) (range)	NPV (%) (range)	PPV (%) (range)
BD GeneOhm	82.1–100	90.6–99.2	96.7.0–100	58.6–94.4
BD Max Cdiff	97.7	99.7	99.7	97.7
Prodesse ProGastro Cd	77.3–100	93.4–99.2	95.9–100	82.8–94.4
Cepheid GeneXpert	94.4–100	93.0–99.2	99.3–100	78.9–94.7
Meridian Illumigene	86.7–98.1	98–100	98.1–99.5	91.8–98.5
Focus Technologies Simplexa[b]	90.1/79.6[a]	93/95.8[a]	NA	NA
Great Basin Portrait[b]	79.6–90.1	93.0–95.8	95.3–98.4	66–81.4
Quidel AmpliVue *C. difficile* Assay	93.6	94.1	NA	NA
Nanosphere Verigene	98.7/91.8[a]	87.6/92.5[a]	99.9/98.5[a]	42.1/67.3[a]

[a] The first result represents comparison to direct toxigenic culture, and the second represents comparison to enriched toxigenic culture.
[b] No publications to date on the FDA platform; data are from the package insert.
[c] Data were compiled from references 57, 60, 87, 89, 96, 107, 122, 130–136, and 139–143.

GeneXpert Infinity, that accommodates 48 or even 80 cartridges in a completely random-access format. Table 5 lists the published performance characteristics for *C. difficile* detection in patient samples (87, 133–136). There have been three published reports on the performance of the Xpert CD/Epi assay. Pancholi et al. found 100% accuracy for detection of all 36 027 strains (137). In a comparison of the Xpert assay to ribotyping, Babady et al. (138) found that 42 of 45 ribotype 027 strains were correctly identified. Both of these studies were conducted in areas of the United States where ribotype 027/NAP1/BI strains are prevalent. In contrast, a study from Australia, where disease caused by ribotype 027 strains is rare, reported false-positive results were related to non-027 isolates with unusual mutations that were amplified by the primers in the Xpert CD/Epi assay (139).

The Illumigene assay (Meridian Bioscience, Cincinnati, OH) is the only FDA-cleared NAAT that is based upon the principle of loop-mediated isothermal amplification (LAMP). This assay detects a conserved 5′ sequence of *tcdA* on the PaLoc by using four primers designed to detect six distinct regions (140). The assay, which has seven manual steps before it is loaded onto the reader, is said to detect toxin A-negative, toxin B-positive strains, as it detects a remnant of the gene in the PaLoc. Features of this assay and its performance characteristics derived from several studies are summarized in Tables 4 and 5 (139–141). One important point to make is that while the test is simple to perform, it does require specific training. There are two critical steps that could lead to a higher-than-expected "invalid" rate if not performed correctly. These include filling of the sample collection brush with the right amount of stool sample and adhering to the specified time between preparing the sample and placing it into the Illumipro-10 instrument (142). Another concern is the potential for spore contamination related to all of the manual processing steps.

Focus Diagnostics Inc. (Cypress, CA) has developed the Simplexa *C difficile* Universal Direct assay. This is a real-time PCR system that targets conserved regions of *tcdB*. There are three steps in this assay. First, the liquid or soft stool samples processed in Tris-EDTA (TE) buffer are heat denatured at 97°C for 10 min. After a reagent preparation step, amplification of *C. difficile* DNA and internal control DNA is performed by using fluorescent bifunctional probe-primers together with reverse primers. The specimens and reagent mixtures are loaded onto a Universal Disc for use on the 3 M Integrated Cycler. The instrument software interprets the results and will suppress them if any of the control samples are invalid. The user examines the amplification curves for each patient result along with the DNA internal control to ensure that results are interpreted properly (143). At the time of preparation of the manuscript, there have been no reported studies using this assay.

The Portrait Toxigenic *C. difficile* assay (Great Basin Inc., Salt Lake City, UT) combines helicase-dependent amplification (HDA) with target detection on silicon chip macroarrays (144) to detect the *C. difficile* toxin B gene. The assay uses a proprietary Hot Start approach—RNase-mediated amplification—to prevent primer artifacts during amplification, which is a problem associated with other isothermal amplification systems (144). The array technology is also unique in that the target DNA is biotin labeled. When the target DNA is captured by probes on the array, hybridization is detected in an enzyme-linked immunosorbent assay (ELISA)-type format by the reaction of each bound target with antibiotin/horseradish peroxidase (HRP) conjugates. Reaction of

subsequently bound conjugates with a precipitable tetramethylbenzidine (TMB) substrate results in signal amplification, which enhances the sensitivity of the assay. After placing a swab into the patient's specimen, the user inserts it into a tube containing extraction buffer, vortexes it, and then filters it by using a 3-ml syringe. Subsequently, 180 µl is delivered into the sample port of the proprietary cartridge of the assay. After the sample port is closed, the disposable cartridge is inserted into the analyzer, and testing begins. The cartridge contains all reagents necessary to perform the test; amplification and detection both occur within a closed system so as to prevent contamination. The internal control detects the *S. aureus nuc* gene. Results are reported qualitatively within 90 min. In a paper by Hicke et al., the analytical sensitivity was reported to be 10 CFU per fecal sample (144). In a small preclinical study (*n* = 130 specimens) performed by those authors, the Portrait test had a sensitivity of 97% and a specificity of 100% compared to the BD GeneOhm Cdiff assay (144). The results of the clinical trial for FDA approval are provided in Table 5.

Like the Illumigene assay, the AmpliVue *C. difficile* assay (Quidel Molecular, San Diego, CA) targets a highly conserved region of *tcdA*. The assay utilizes helicase-dependent isothermal amplification in a self-contained handheld cassette for amplicon detection. In brief, a swab is used to inoculate a small amount of liquid sample into a dilution tube. Diluted sample (50 µl) is added to a lysis buffer tube, which is heated in a heating block at 95°C. After the 10-min lysis step, 50 µl of sample is added to a reaction tube and heated for 60 min at 64°C. The biotinylated single-stranded DNA amplicons bind to capture probes during the reaction. After incubation, the reaction tube is placed into the amplicon cartridge, and the cartridge is placed into the hinged detection chamber of the handheld cassette. A lateral flow strip within the cassette uses streptavidin-conjugated color particles to visualize the dually labeled probe-amplicon hybrids. These appear as pink-to-red lines on the cassette. There is also a line that corresponds to the process control DNA that must be visible for any valid result (146).

The Verigene *C. difficile* test (Nanosphere Inc., Northbrook, IL) is the most recent assay to obtain FDA clearance. This assay detects both *tcdA* and *tcdB* as well as *cdt* and the deletion at nucleotide 117 on *tcdC*. The Verigene assay requires a sample processing step of a swab placed into the fecal sample, followed by inoculation of the specimen in buffer into the test cartridge. The test cartridge contains a reagent pack, which is a single carrier that sits atop the array, a glass slide that is solid support for the capture oligonucleotides. The cartridge contains all of the reagents needed for amplification and hybridization for each test, and it also captures all waste generated during testing. The testing within the cartridge is performed with an instrument called the Verigene Processor SP.

The Verigene assay has several unique features. The extracted DNA is sheared by a sonication process into 300- to 500-bp fragments to facilitate the hybridization steps. In step 1, the sheared DNA is hybridized to single-stranded DNA capture oligonucleotides on the array and simultaneously to sequence-specific mediator oligonucleotides that detect single-copy DNA regions of each target. In the second hybridization reaction, gold nanoparticles bind to captured target nucleic acid/mediator oligonucleotide complexes. After a wash step, silver signal amplification of the gold nanoparticle probes occurs. A final washing step removes unre-

acted reagents. When testing is completed, the glass slide is removed and placed into the reader for interpretation. Results are reported qualitatively (147). To date, there have been no publications on this assay, but the results from a five-center clinical trial are summarized in Table 5. Like the GeneXpert *C. difficile* assay, this test may presumptively recognize ribotype 027 strains because it detects the nucleotide 117 deletion on *tcdC* and the binary toxin genes. Although there are no publications available, the data in the package insert show that compared to PCR ribotyping of the isolates recovered from the enriched culture, there was 91.4% positive agreement (147). The assay failed to amplify four specimens that were positive for ribotype 027 by enriched culture (147). One specimen was positive for *tcdA* and *tcdB* but was negative for *cdt* and Δ117, from which a ribotype 027 strain was recovered upon culture (147). Five specimens tested positive for *cdt* and Δ117 by the Verigene assay, but the ribotype recovered was not ribotype 027 (147).

(i) Impact of nucleic acid amplification tests on clinical care. While molecular assays have certainly improved the detection of *C. difficile* in fecal samples, there are numerous questions that have been raised regarding their clinical utility. Some concerns relate to the biology of *C. difficile* and how detection of the genes that encode toxins A and B correlate with expression of toxin. For example, is the gene always expressed, or, given the heterogeneity of *C. difficile*, will strains emerge that are not detected by a particular assay? In terms of the former question, in the majority of the clinical trials, compared to toxigenic culture, there are a few situations where the assays failed to detect a toxigenic organism recovered from a stool sample. It is unclear, however, whether these are related to concentrations of organisms below the limit of detection of an assay, specimen handling problems, or user errors. Few studies have included sequencing or other methods to assess failure of primer-probe binding or problems with completion of amplification cycles. All of the assays discussed above have built-in controls to assess inhibition, and this is one parameter that can be monitored in clinical laboratories as a component of ongoing assay validation.

A few studies have focused on variability in test performance based upon the ribotype of the infecting strain. One study found that the GeneXpert and nonamplified methods, such as an EIA for GDH antigen detection, had equivalent performances for detection of ribotype 027 strains (118). Conversely, significant differences in the performances of the EIAs for toxin A/B and GDH were seen compared to PCR for non-027 isolates (118). However, other studies have not seen this discrepancy based upon strain type (120). It is important to note that almost all of these studies suffered from small numbers of some ribotypes. The reasons for variability in assay performance by strain type for the nonamplified tests are hypothetically related to differences in toxin production among those strains, a cited advantage to molecular testing (121). More data on the impact of strain type on assay performance are needed. The reader is referred to the sections on GDH and epidemiology for more discussion of this topic.

As the circulating strain types causing disease change, it may be important to periodically assess both analytical and clinical performance of molecular tests as a component of ongoing validation. It is unclear if this is the responsibility of the vendor, the user, or public health institutions. Those states, countries, or regions that have an existing surveillance program in place could alert clinical laboratories when a new, predominant strain enters a community. This information could be used to assess assay per-

formance. Since all assays have focused on conserved regions of the toxin A and B genes in the PaLoc found in strains of all ribotypes, it seems less likely that failure to detect a strain in clinical samples is related to mutations within the target sequences, although this remains a theoretical possibility (121).

More important questions concern the clinical utility of NAATs, particularly the specificity and positive predictive values, given that a gene and not an actual toxin is detected. Several studies demonstrating that inappropriate test ordering can certainly impact the specificity of these tests have emerged. In a study by Dubberke and colleagues (96), 36% of patients for whom a *C. difficile* test was ordered did not have clinically significant diarrhea; moreover, 20% of patients had recently taken a laxative. When clinical parameters were used to assess the performance of various test methods, the NAATs had the highest sensitivity but the lowest specificity compared to other modalities (96). In a study to assess the impact of education on *C. difficile* test ordering practices, Redding et al. (273) likewise observed that the presence of diarrhea was recorded in the chart for only 50% of patients for whom testing was ordered, and 44% of patients had received a laxative in the prior 48-h period. Educational intervention succeeded in improving documentation to 71% and reducing testing of patients on laxatives to 21% (Redding et al., submitted).

Although there are few studies that include clinical data when assessing performance characteristics of *C. difficile* testing, the existing data highlight that although NAAT assays for *C. difficile* in fecal specimens are analytically sensitive and specific, clinical specificity may not be optimal; that is, detection of *C. difficile* in a specimen does not always equal disease, as NAAT assays can be positive in both colonization and disease states. It seems plausible that the future of *C. difficile* diagnostics will include detection of not only *C. difficile* but also a biomarker that correlates with active *C. difficile* infection. While several biomarkers have been evaluated (such as fecal lactoferrin and various cytokines [see "Adjunctive tests and biomarkers for diagnosis of *C. difficile* disease" below), to date, an optimal adjunct assay for NAAT testing has not been established. This will likely be an area of active investigation in the coming years.

Just as an example of the great interest in establishing improved diagnostics for *C. difficile* infection, one recent study reported that a dog (2-year-old male beagle) under the care of a professional trainer was able to "sniff out" *C. difficile* in stool samples (148). When the dog was presented with positive and negative stool samples, the dog's sensitivity and specificity for detection of *C. difficile* were 91 to 100% and 83 to 98%, respectively (148). However, it seems unlikely that a sniffing dog will be broadly adopted as the adjunctive assay of the future; not only do logistical concerns exist with keeping a dog in the microbiology laboratory, this would also be completely uncharted territory for regulatory bodies such as the College of American Pathologists (CAP) and the FDA.

Since molecular assays cost up to 2- to 3-fold more than EIAs, the issue of cost-effectiveness has been raised by laboratorians and administrators. To answer this question, the impact on treatment and infection control activities should be considered. A few studies are beginning to emerge to address the impact on pharmacy costs and length of stay in isolation, with favorable results. Peterson and Robicsek (149) were the first to hypothesize that implementation of a NAAT would lead to fewer tests ordered due to enhanced accuracy, which might offset the increased costs of these platforms. Tenover et al. (121) likewise created a hypothetical

model based upon testing of 1,000 patients, assuming a 10% prevalence of CDI and using published performance characteristics of various test methods. NAAT testing alone, compared to a toxin EIA alone and to various two-step algorithms using GDH detection, resulted in the largest number of patients with disease who would be placed in isolation within 24 h, the largest number of patients who would be removed from isolation more quickly because the rapid NAAT would detect them more rapidly, and the smallest number of patients with false-negative tests who would not be placed in isolation and consequently who would continue to spread the organism within the hospital environment (121).

In actuality, several studies clearly note that *C. difficile* rates doubled when laboratories made the switch from an EIA to molecular testing or incorporating a molecular test into a testing algorithm. This could have a negative impact on public health reporting if the method of testing is not considered (150–153). One study hypothesized that this may be a disincentive to eliminating less sensitive EIAs (151). After a period of increase, rates declined, suggesting better case definition leading to decreased transmission (151). The latter scenario played out at the Johns Hopkins Hospital (JHH). In 2010, the laboratory switched completely to a NAAT that was implemented twice daily 5 days per week and offered once per day on weekends. In the period immediately after implementation, *C. difficile* transmission rates appeared to increase from 11.5 cases/10,000 patient days to close to 15 cases/10,000 patient days for calendar year 2010 (Lisa Maragakis, JHH, personal communication). Rapid test availability allowed for improved isolation. Additionally, the apparent increase in transmission was used to campaign for better hand hygiene and compliance with infection control practices, such as enhanced environmental cleaning. By the second quarter of 2012, the transmission rates were among the lowest seen since 2007: 9 cases/100,000 population (Lisa Maragakis, JHH, personal communication).

In a paper by Catanzaro and Cirone, the authors performed a retrospective study comparing the impact of the switch from a toxin A/B EIA to a PCR method (154). Prior to implementation of the PCR test, the practice was preemptive isolation of symptomatic patients with the caveat that three negative EIA results were required to remove patients from isolation. The latter rule was based upon poor performance of the EIA in their environment, which led to clusters of transmission in some units (154). When the laboratory switched to the PCR test, preemptive isolation was discontinued upon report of one negative test result (154). Postimplementation, the authors observed a significant decrease in health care-associated CDI (4.4 cases/10,000 patient days versus 0.9 cases/10,000 patient days; $P = 0.02$), a reduction in patient isolation days (1,022 versus 364; $P = <0.00001$), fewer tests ordered ($P = 0.02$), and a reduction in the duration of empirical metronidazole therapy for patients with negative *C. difficile* tests ($P = 0.02$) (154). In a study by Sydnor et al. (155), replacement of a two-step algorithm using GDH detection and toxin EIA with an algorithm using GDH detection with PCR resulted in a 54% reduction in empirical antimicrobial use. Unfortunately, those authors did not assess cost savings in either study.

Others are using the assays that detect ribotype 027/NAP1 strains to control the use of more expensive antimicrobial agents such as fidaxomicin. Based upon the original modified intention-to-treat and per-protocol study analyses, fidaxomicin was noninferior to vancomycin with respect to clinical cure rates (156). Significantly fewer patients in the fidaxomicin treatment group had recurrences, unless they were infected with NAP1/ribotype 027 strains, in which case there was no difference between the treatment arms (156). Currently, there are no recommendations to alter infection control practices or to change therapy on the basis of detection of a specific ribotype alone (see the discussion on strain types and disease severity elsewhere in this review).

Few papers have addressed the comprehensive laboratory costs associated with various test methods, and none have measured the impact on overall hospital costs. In one study comparing two-step algorithms predicated upon a GDH result incorporating a cytotoxin test or the Xpert *C. difficile* assay for GDH-positive specimens, performing the Xpert test alone was less expensive when labor costs (accessioning, test performance, and reporting of results) were considered (138). In a study by Larson et al. (104), the authors projected that in making the diagnosis of *C. difficile* 1 day earlier in cases missed by EIA, PCR testing alone could save $200,000 annually by avoiding the costs of repeat testing. Additional research is needed to assess the overall cost-effectiveness of NAATs for the diagnosis of *C. difficile* disease.

Evolution of Testing Methods

As suggested above in the sections describing diagnostic assays for *C. difficile*, the methods used by clinical laboratories have evolved over the last 2 decades, from CCCNA to toxin EIAs to NAATs. Table 6 summarizes the most common assays used for *C. difficile* detection by clinical laboratories subscribing to the CAP proficiency testing program from 2001 to 2012. These data were included with permission from the CAP Microbiology Resource Committee. One of the major differences noticed over time is that a number of laboratories were using an EIA that detected only toxin A in the early 2000s, but gradually, that changed, such that no laboratories were using this type of assay. It is interesting to note the shift away from CCCNAs and EIAs toward the use of molecular testing methods; in 2012, nearly one-third of laboratories reported using a molecular method. Although the methodologies have changed, the overall performance of laboratories in these proficiency testing challenges, as well as the number of laboratories participating in these challenges, has remained relatively stable over this time period (Table 7).

Adjunctive tests and biomarkers for diagnosis of *C. difficile* disease. A variety of fecal biomarkers of inflammation have been developed over the last several decades in an attempt to distinguish inflammatory versus noninflammatory causes of diarrhea and other gastrointestinal complaints. These assays have been embraced by gastroenterologists as noninvasive mechanisms for screening of patients, particularly for inflammatory bowel disease (IBD), where they have been shown to have some utility. Their usefulness in the diagnosis of *C. difficile* disease is less clear. This section only briefly touches upon this subject.

(i) Fecal lactoferrin. Lactoferrin is an iron binding glycoprotein found in neutrophils and in secretions such as breast milk, and therefore, this marker may be present in feces of children who are breast fed, reducing its utility as an enticing marker for bacterial enteritis in children <2 years of age (157). This protein is released following neutrophil activation, and the concentrations in stool and other fluids are proportional to the number of neutrophils recruited (158). Fecal lactoferrin is resistant to proteolysis and is not degraded by intestinal bacteria (159).

Fecal lactoferrin was first measured in stool samples in 1992 by a latex agglutination assay developed by Guerrant et al., who as-

TABLE 6 Summary of CAP survey results for the most common assays used for *C. difficile* testing, 2001 to 2012

No. (%) of participants using method (columns Bartels through Meridian Illumigene)

Yr	Survey ID	Total no. of participants	Bartels cytotoxicity assay	BD ColorPac Toxin A	bioMérieux Vidas	Meridian Premier Cytoclone A & B	Meridian Premier Toxin A	TechLab/Wampole Toxin A/B	Thermo BioStar OIA	Meridian Immuno-Card Toxins A & B	Meridian Premier Toxins A & B	Remel Xpect Toxin A & B	TechLab/Wampole Tox AB II	TechLab/Wampole Toxin A/B Quik Check	TechLab/Wampole C. Diff Quik Chek Complete	BD GeneOhm	Cepheid Xpert	Meridian Illumigene
2001	D-C	2,384	33 (1.3)	137 (5.7)	231 (9.6)	204 (8.5)	261 (10.9)	309 (13.0)										
2002	D-A	2,127	31 (1.5)	137 (6.4)	220 (10.3)	226 (10.6)	214 (10.1)	274 (12.9)										
2003	D-B	2,207	25 (1.1)	125 (5.7)	179 (8.1)	353 (16.0)	53 (2.4)	315 (14.2)										
2004	D-B	2,021	22 (1.1)	111 (5.5)	142 (7.0)		93 (4.6)	292 (14.4)	213 (10.5)		573 (28.4)							
2005	D-A	1,972	17 (0.9)	81 (4.1)	127 (6.4)		71 (3.6)	257 (13.0)	197 (10.0)		545 (27.6)	92 (4.7)						
2006	D-A	2,041	12 (0.6)	44 (2.1)	83 (4.1)		32 (1.6)	248 (12.1)	141 (7.0)	314 (15.3)	563 (27.6)							
2007	D-A	2,304	12 (0.6)	32 (1.4)	54 (2.3)				78 (3.3)	449 (19.4)	570 (24.7)	142 (6.2)	258 (11.2)	79 (3.4)				
2008	D-A	2,096	10 (0.5)	15 (0.7)	37 (1.8)				54 (2.6)	467 (22.3)	488 (23.2)	150 (7.2)	195 (9.3)	145 (6.9)				
2009	D-A	2,170	10 (0.5)	10 (0.5)	33 (1.5)				13 (0.6)	506 (23.3)	492 (22.7)	155	189 (8.8)	160 (7.3)				
2010	D-A	2,239		15 (0.7)	42 (1.9)					489 (21.8)	438 (19.6)	137 (1.6)	172 (7.7)	153 (6.8)	126 (5.6)			
2011	D-B	2,324			62 (2.7)					484 (20.8)	367 (15.8)	121 (5.2)	120 (5.2)	131 (5.6)	340 (14.6)	31 (1.3)	253 (10.9)	85 (3.7)
2012	D-A	2,189			37 (1.7)					360 (16.4)	224 (10.2)	83 (3.8)	78 (3.6)	96 (4.4)	341 (15.6)	48 (2.1)	332 (15.2)	267 (12.2)

TABLE 7 Summary of CAP results for *C. difficile* testing, 2001 to 2012

Yr	Survey ID	Total no. of participants	Intended response	No. (%) of participants with correct response
2001	D-C	2,384	Positive	2,220 (93.1)
2002	D-A	2,127	Negative	2,090 (98.3)
2003	D-B	2,207	Positive	2,162 (98)
2004	D-B	2,021	Negative	1,979 (97.8)
2005	D-A	1,972	Negative	1,919 (97.3)
2006	D-A	2,041	Positive	1,970 (96.5)
2007	D-A	2,304	Negative	2,235 (97)
2008	D-A	2,096	Positive	2,077 (99.1)
2009	D-A	2,170	Positive	2,156 (99.4)
2010	D-A	2,239	Positive	2,222 (99.2)
2011	D-B	2,324	Negative	2,310 (99.4)
2012	D-A	2,189	Negative	2,114 (96.6)

sessed the ability of the assay to detect patients with *Shigella* infection (160). Compared to uninfected controls, 96% of patients with shigellosis had fecal lactoferrin titers that ranged from 1:200 to ≥1:5,000 (160). This same group also showed that 92% of patients with *C. difficile* enteritis had elevated latex agglutination titers of fecal lactoferrin of >1:50, with 50% having titers of >1:400 (161). Since then, others have reported on the utility of fecal lactoferrin testing for assessing severity of *C. difficile* infection (162), as a marker for infection associated with moxifloxacin-resistant strains (163), and in testing algorithms as a first step in deciding which patients with diarrhea should be tested for *C. difficile* (159). It has also been used to determine the clinical significance of results using multistep algorithms that involve GDH screening and PCR confirmation (164). In the latter scenarios, lactoferrin levels were higher in patients who were GDH positive/toxin positive than in GDH-positive/toxin-negative/PCR-positive or GDH-negative patients (164). In a study by van Langenberg et al., the authors reported a negative predictive value of 92% when the manufacturer's cutoff was lowered from 7.25 μg/ml to 1.25 μg/ml (159). More prospective studies specifically targeting patients with *C. difficile* disease need to be performed before firm recommendations on the use of this test can be made.

(ii) Fecal calprotectin. Calprotectin is a calcium binding protein found within the cytosol of neutrophils, where it accounts for approximately 60% of their cytoplasmic protein content (158). Under inflammatory conditions of the intestinal tract where neutrophils accumulate, calprotectin is excreted in stool and is resistant to bacterial degradation (158). Studies have shown that it is stable at room temperature in feces for up to 7 days (158). In studies of patients with IBD, cutoffs have been established to differentiate functional from organic bowel disease; values of <50 μg/g indicate functional disease, and values of >150 μg/g of calprotectin are predictive of organic disease and highlight patients who should be referred for colonoscopy (158). In those situations where the values fall between 50 and 150 μg/g, other causes of inflammation, such as infections with enteric pathogens, should be considered (158). It should be noted that nonsteroidal anti-inflammatory agents can also raise fecal levels of calprotectin because of mucosal damage to the small bowel, which recruits neutrophils to the site (158).

Few studies have been performed to evaluate the utility of calprotectin in the evaluation of patients with acute diarrhea. Shastri et al. performed a large, prospective, multicenter, case-controlled

study to evaluate the accuracy of fecal calprotectin, fecal lactoferrin, and fecal occult blood testing of patients with suspicion of acute enteritis, including *C. difficile* infection (165). Initially, 2,185 patients from three centers were evaluated by routine bacterial stool culture and a *C. difficile* toxin A and B EIA. Of this large cohort, 9.2% of patients (*n* = 200) were positive for a bacterial pathogen and were age and sex matched to a control group that had negative results (165). Commercial assays were used to perform a quantitative fecal calprotectin test (Immunodiagnostik ELISA kit; Immunodiagnostik AG, Bensheim, Germany), a qualitative fecal lactoferrin test (IBD-Chek; TechLab, Blacksburg, VA), and fecal occult blood tests (Haemoccult; Beckman Coulter Inc., Fullerton, CA) (165). There was an overlap of fecal calprotectin levels between the pathogen-positive patients and the control group (0 to 994 mg/liter and 0.1 to 204.7 mg/liter, respectively). Compared to the other tests, the fecal calprotectin specificity and positive and negative likelihood ratios were statistically better in predicting patients with infectious diarrhea (165). Fecal calprotectin levels were highest for the 87 patients with *C. difficile* diarrhea compared to patients with other infectious causes of diarrhea (165). In this group, the sensitivity of the test with a cutoff value of >15 mg/liter was 82.8% (165); however, this value is below the level of acceptability to be used alone as a screening test. The FDA-approved assay available in the United States has an indication for inflammatory bowel disease.

In summary, both fecal lactoferrin and calprotectin are nonspecific markers of intestinal inflammation, and while studies demonstrate that levels of these markers may be significantly elevated in patients with *C. difficile* disease, the sensitivity is too low in most studies to recommend their routine use for screening of patients. However, it does appear to be the case that the absence of fecal lactoferrin or fecal calprotectin is inconsistent with an inflammatory cause of diarrhea.

(iii) Cytokine analysis. There are no commercial assays available for measuring cytokine levels in fecal specimens. Some investigators have shown the correlation of elevated levels of IL-1β and IL-8 in active disease, particularly in cases of moderate to severe infection (162, 166, 167), with subsequent decreases in levels of these cytokines in stool when the patient recovers from acute infection (166). In a study by Jiang et al., the authors demonstrated that a single-nucleotide polymorphism (SNP) (251 A/A) in the IL-8 gene is associated with increased susceptibility to *C. difficile* disease (167). Cytokine analysis of fecal specimens may be an area to be pursued in future studies with respect to assessing severity of infection, potentially to resolve whether a positive NAAT assay is associated with disease or colonization, and perhaps to monitor a patient's response to treatment.

Algorithmic approaches. As alluded to in the sections above, although NAATs for *C. difficile* detection are more sensitive than toxin EIAs and can be used as stand-alone tests, they are more costly than EIAs. The amount of technologist time required to perform the molecular assays can also be longer than for EIAs, depending on the method used. To address these issues, some laboratories have adopted two- or three-step algorithmic approaches to *C. difficile* testing. In the algorithmic approach, samples are screened by using a GDH assay, and if the GDH assay is negative, the specimen is reported as negative without additional testing. If the specimen is GDH positive, confirmatory testing must be performed. In the two-step algorithm, the confirmatory test is typically a molecular assay, and the sample is reported as

either positive or negative based on the molecular test result. In the three-step approach, samples are tested for GDH, and if positive, they are tested by using a toxin EIA. Toxin-positive samples can be reported as positive, but GDH-positive/toxin-negative samples are then tested by using a molecular test. Alternatively, the samples can be screened by using a GDH/toxin combination EIA, but since the toxin EIA component of these assays has a sensitivity comparable to those of stand-alone tests (i.e., inadequate sensitivity), GDH-positive/toxin-negative samples should be reflexed to testing (i.e., automatically tested without a physician order when another test is positive in a multitest algorithm) via a molecular method (87, 90, 91, 100, 122–124). These multistep approaches can be especially enticing to laboratories without the equipment or expertise for molecular testing, in order to minimize the number of specimens sent to a reference laboratory for this analysis.

Although algorithmic testing represents a potential cost savings to the laboratory, there are a number of factors to consider when evaluating this approach. The first factor is the prevalence of GDH-positive specimens in a given population. For example, in pediatric patients (discussed below), the rate of GDH-positive specimens may be very high, necessitating the use of two or more assays on a large proportion of specimens submitted. In these cases, cost savings may be negligible. Laboratories will need to consider the clinical impact of the prolonged turnaround time to reporting for specimens requiring multiple tests. Some other practical considerations include the need for inventory, training of staff, quality control, and maintenance of procedures and competency for multiple assay types. There is also the caution that GDH assays appear to be slightly less sensitive than NAATs. These issues are not trivial and must be taken into consideration. Thus, each laboratory must evaluate the utility of multistep algorithms for their patient population.

(i) The European perspective. NAAT assays for PCR detection are highly sensitive for *C. difficile* detection, but they do not differentiate between active infection and asymptomatic carriage. As the antimicrobial agents used to treat *C. difficile* infection can also induce *C. difficile* disease, unnecessary antimicrobial therapy as a result of false-positive assay results is not without consequence. The National Health Services (NHS) laboratories in England are now following a different protocol from what is used in most of the United States. The NHS algorithm uses either a molecular assay or a GDH assay to screen for the presence of *C. difficile*, followed by a "good" or "relatively sensitive" EIA to confirm the diagnosis (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/146808/dh_133016.pdf.pdf [accessed 25 April 2013]) (168). The rationale for this is that the GDH and molecular assays have high NPVs and can be used to expedite the exclusion of *C. difficile* infection. It has been proposed by the NHS that patients who are positive for *C. difficile* by a molecular assay but who are negative for toxin by EIA methods can be declared "potential *C. difficile* excretors," and the decision may be made to apply isolation precautions for *C. difficile* for these patients, but they do not require treatment for disease; other causes of the patient's diarrheal illness should be considered at this point (168, 169). This approach of a NAAT test followed by a toxin EIA is very uncommon in the United States. At the time of preparation of this article, the data supporting these recommendations are under review for publication.

Antimicrobial susceptibility testing. The most commonly pre-

scribed antimicrobial therapy for *C. difficile* infection is metronidazole or oral vancomycin. As the concentrations of these drugs in the feces are typically high, treatment failures are rarely thought to be due to resistance of the organisms to the antimicrobial therapy (34, 170). However, metronidazole concentrations in the stool can be variable depending on the consistency of the stool and the amount of inflammation in the gut. Some studies have demonstrated that more treatment failures occur with metronidazole therapy than with vancomycin and that higher rates of recurrence are observed in severely ill patients whose *C. difficile*-associated disease (CDAD) is treated with metronidazole (171, 172). In addition, there have been sporadic reports of *C. difficile* isolates with increasing MICs of metronidazole (173–176). The method currently recommended by the Clinical and Laboratory Standards Institute (CLSI) for *C. difficile* antimicrobial susceptibility testing is the agar dilution method using Brucella agar supplemented with hemin, vitamin K, and 5% (vol/vol) laked sheep blood (177). For metronidazole and *C. difficile*, an MIC of <8 μg/ml is considered susceptible. The agar dilution method is very technically demanding and labor-intensive. While this method may be appropriate for epidemiological studies, it is difficult to implement for routine use in the clinical laboratory. Several studies have evaluated the use of disk diffusion and/or Etest/gradient diffusion methods, as these are more amenable for routine use; however, there is much variability in the methods that laboratories use to perform these tests, including the use of both 5-μg and 30-μg disks, different medium formulations, and different incubation times (178–180). No CLSI standardization or guidelines for disk diffusion or Etest for *C. difficile* have been established. In general, these methods exhibit good agreement with one another and with agar dilution methods (178), although the MICs obtained by using the Etest are slightly lower than those demonstrated by agar dilution (179, 180).

EVOLVING EPIDEMIOLOGY

Clinical Implications of Strain Typing

Epidemiology. The epidemiology of *C. difficile* has been in a state of flux over the last 2 decades. There has been an increase in the incidence and severity of CDI in North America and Europe since the early 2000s, and in 2003, the first reports emerged from Quebec, Canada, of a 4-fold increase in CDI rates (26, 181, 182). During 2005, an increase in the rate of *C. difficile* infection was noticed around North America, with a large number of cases being attributed to NAP1/ribotype 027 strains, which exhibited high-level resistance to fluoroquinolones; this trait is thought to be one of the main factors contributing to the success of the rapid spread of this strain type, although the increased toxin production observed *in vitro* has also been postulated to contribute to the high rate of outbreaks of this strain (29, 30, 46–50). Retrospective analysis revealed that ribotype 027 strains were isolated as early as 1958. It appears that different lineages evolved with phenotypic distinctions resulting in different antimicrobial susceptibility patterns and toxin production. Ribotype 027 has an 18-bp deletion and a frameshift mutation due to a single-base-pair deletion at position 117 of *tcdC* (183) that results in a truncated TcdC that lacks function. TcdC is a negative regulator of toxin A and toxin B production, and the resultant hyperproduction of toxin is thought to contribute to increased virulence. Increased sporulation capacity has been hypothesized to contribute to increased transmission of this strain type (46, 49). In contrast, other studies have shown that

ribotype 027 is not more virulent than other strain types (184, 185) and that the sporulation rate and toxin production rate are not linked to strain type (170, 186, 187), so this remains an area of controversy.

The increased incidence and severity of *C. difficile* disease have pushed *C. difficile* to the forefront as an important public health problem (34, 182, 188–190). As a result, accurate and timely identification of patients with *C. difficile* infection has been an area of focus for clinical laboratories in recent years.

In addition to the clinical importance of the evolving epidemiology of *C. difficile*, changes in circulating strain types are also of importance for clinical laboratories. For example, with the emergence of toxin A-negative strains associated with severe clinical disease, assays that rely solely on detection of toxin A are generally no longer used (36, 84, 191) (Table 6). It has also been reported that assay performance might vary depending upon the strain type (118).

Community-acquired *C. difficile* infection. Although *C. difficile* is the number one cause of hospital-acquired diarrhea, it is not exclusively a nosocomial pathogen (34, 192). Numbers of cases of community-acquired and community-associated infection are increasing worldwide; around the world, up to 25% of cases are community acquired, and these patients may not have the traditional risk factors for CDI, such as advanced age, antibiotic exposure, and medications to suppress gastric acid (188, 192–195). A pilot study was recently conducted whereby whole-genome sequencing (this method is discussed in more detail below) was performed in almost real time to evaluate *C. difficile* transmission. In this analysis, samples from all of the cases detected over a 6-week period in a single hospital were sequenced and compared with local strain sequences from the previous 3 years. This analysis suggested community transmission, which was not previously suspected (196).

Transmission in food has been proposed as a plausible and enticing explanation for community-acquired disease. While *C. difficile* has been isolated from food, food-borne transmission of disease has not been demonstrated, even though the predominant strain types isolated in food are ribotypes 027 and 078, strains well established to cause human disease (197–201). One recent study reported *C. difficile* recovery from 5 out of 119 seafood and fish samples from a grocery store (all of which were of ribotype 078) (200); in contrast, a large survey that tested for *C. difficile* in 1,755 retail meat samples from across the United States (ground beef, ground turkey, chicken breast, and pork chop) found that no *C. difficile* was isolated from any of these specimens (198), which suggests a low prevalence of contaminated meat products. Thus, the true impact of food as a vehicle for *C. difficile* transmission remains to be clarified.

Strain Typing Methods

A number of characteristics are important for strain typing. Some of the most important properties are the ability of the method to group related isolates, the ability to differentiate unrelated isolates, typeability (i.e., the ability to resolve isolates as similar or distinct in an unambiguous manner), reproducibility (intra- and interlaboratory), and cost-effectiveness, and if possible, the interpretation should be objective (202). The different typing methods employed embody various degrees of each of these characteristics. The "discrimination index" (*D*) is a metric that has been devised to compare the abilities of different typing methods to discriminate unique strains as different and is based on Simpson's index of diversity (203, 204). Values for *D* range from 0 to 1, and the closer

the value is to 1, the more discriminatory the method. Historically, strain typing methods relied on phenotypic patterns such as antimicrobial susceptibility profiles, serotyping with slide agglutination methods, immunoblotting, or bacteriophage patterns. Today, most typing methods for microorganisms are based on molecular analysis.

There has been great interest in strain typing of isolates of *C. difficile* for a number of reasons (205). The first is for epidemiological purposes at a global level, both to evaluate the strain types circulating in different parts of the world and to evaluate the evolution of these strain types. At the local level, strain typing can be used to track transmission and outbreak events. From the perspective of diagnostic testing, it has been proposed that the performance characteristics of some of the available assays may vary depending upon the strain type, which could be of great clinical and public health importance (118). As more and more laboratories make the shift to molecular methods for *C. difficile* detection, it will be important to monitor trends in the circulating strain types to ensure that *C. difficile* variants that may arise have conserved nucleic acid sequences in the regions targeted by these molecular assays, or we may see a shift in the sensitivity of the molecular assays over time.

From the perspective of patient care, it is postulated that strain typing might be important in some circumstances, although routine strain typing of all isolates is not currently indicated. Some strain types may be more correlated with risk of recurrent disease, although more investigation is needed to confirm this (2). In addition, new antimicrobial therapies for *C. difficile* are emerging, such as fidaxomicin. Fidaxomicin is much more costly than traditional therapies for *C. difficile*, such as metronidazole or vancomycin. Early studies evaluating fidaxomicin suggested that the efficacy of this drug and/or risk of relapse on this drug may be variable with the *C. difficile* strain type. For example, Louie et al. reported that the rate of recurrence of non-NAP1 *C. difficile* infection was significantly lower with fidaxomicin (156). Considering the cost of this drug, if these initial findings are replicated in other studies, it could result in an increased demand for real-time strain typing to determine which patients are best suited to receiving this therapy.

In the future, there may be a role for strain typing of isolates from patients with recurrent *C. difficile* disease in order to ascertain if these patients have a relapse of the strain originally causing the infection or if they have acquired or become reinfected with a new strain from the environment. It is possible, with more data, that this type of information may change how clinicians caring for these patients stratify treatment options for individuals with recurrent disease.

A number of different strain typing methods for *C. difficile* have been described and evaluated. To date, only one major study has compared a number of these techniques simultaneously: Killgore and colleagues evaluated restriction endonuclease analysis, pulsed-field gel electrophoresis (PFGE), PCR-ribotyping, multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA), amplified fragment length polymorphism analysis, and surface layer protein A gene sequencing typing (206). The analysis included a relatively small sample set of 42 isolates from four countries. While this study was biased toward NAP1 strains, it did provide an assessment of how these typing methods related to one another. The strain typing techniques for *C. difficile* that are used most frequently clinically or for outbreak investigations are described in more detail below.

Pulsed-field gel electrophoresis. PFGE was one of the first molecular typing methods used for *C. difficile*, and it is typically still considered the standard for *C. difficile* typing in North America. PFGE involves digestion of genomic DNA with a restriction enzyme that is a "rare cutter," or infrequently cuts the genomic DNA; for *C. difficile*, SmaI is commonly used (207). The resultant DNA fragments are separated by using agarose gel electrophoresis, but the electric field is repeatedly switched in three different directions during this process (pulsed field). This allows resolution of large fragments of DNA that would not be possible using conventional agarose gel electrophoresis. The resulting banding patterns are referred to as "North American pulsed-field" (NAP) types. Strains with >80% similarity are usually regarded as a single pulsotype, although there can be a great deal of subjectivity in interpretation of the banding patterns, especially when differences between the test strain and the reference strains are slight. In the typing method comparison study by Killgore et al., the *D* value for PFGE was reported to be 0.843 (206).

Ribotyping/PCR-ribotyping. Ribotyping is the strain typing method used most frequently for *C. difficile* in Europe. This method is based on amplification of the intergenic spacer (ITS) region between the 16S and 23S rRNA genes. This operon has several copies in the *C. difficile* genome, and the different copies vary in length. Therefore, a single primer pair can result in a pattern of bands, usually ranging from 200 to 700 bp (208, 209). The bands can be resolved, visualized, and compared on an agarose gel or by using commercial kits or analysis software (206, 208–210).

Two major primer sets have evolved for PCR ribotyping: the "United States" primers and the "United Kingdom" primers (206). The primers described by Stubbs et al. appear to be more discriminatory for *C. difficile* strains than the primers described by Bidet et al. (208, 209). In general, PCR-ribotyping exhibits good discriminatory power but may not be able to differentiate between strains of the closely related ribotypes 027, 106, and 017 (211). In the method comparison study by Killgore et al., the *D* value for PCR-ribotyping using the U.S. method was 0.700, and that using the United Kingdom method was 0.688 (206).

It can be difficult to compare banding patterns between laboratories if analysis software is not used. To circumvent the issues related to manual interpretation of banding patterns, PCR-ribotyping has recently been adapted to high-resolution capillary gel electrophoresis, which has improved the reproducibility of ribotype interpretation (212). In the United Kingdom, work is ongoing to standardize ribotyping protocols across the country using this method.

In the United States, no natural or mandated surveillance system is in place for *C. difficile*. In contrast, England has instituted a *C. difficile* reporting program, whereby isolates are submitted to a central laboratory and a portion of these isolates are ribotyped to monitor epidemiological trends. Figure 1 depicts the presence of different ribotypes in England by quarter from 2007 to 2009 (213). In North America, ribotype 027 is predominant, although ribotype 078 is emerging. New ribotypes of clinical significance continue to emerge; two of the newly described ribotypes, 176 and 198, are closely related to ribotype 027 (and can be mistaken for ribotype 027, depending upon the methodology used) (214). These ribotypes all cluster together by multilocus sequence typing, suggesting coevolution of these strain types and a common ancestry. The fact that North America relies largely on PFGE and Europe relies largely on PCR-ribotyping for strain typing and strain
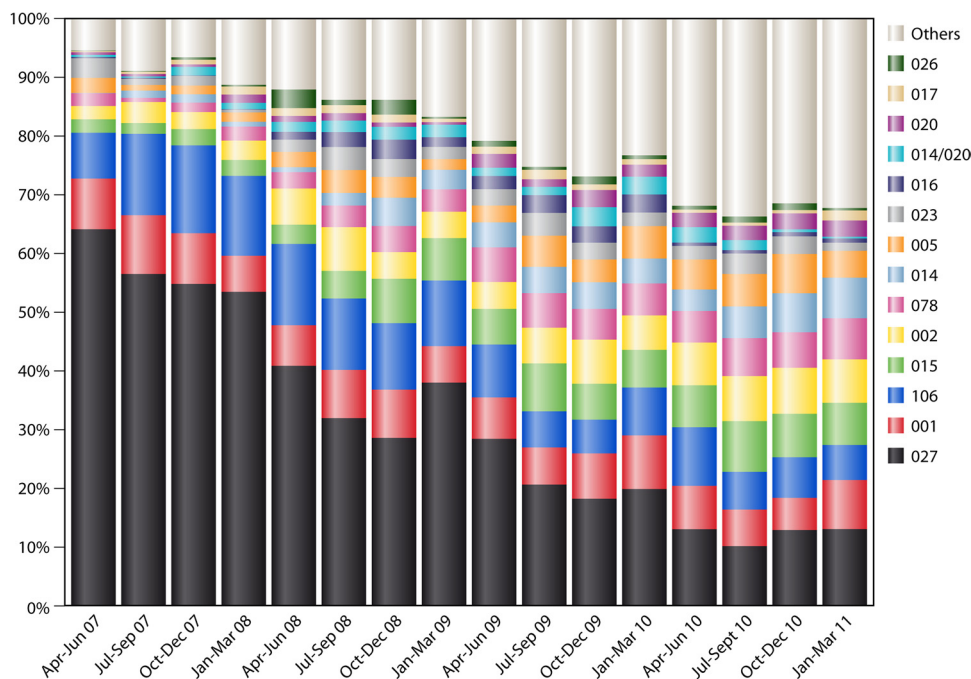
FIG 1 Prevalence of *C. difficile* ribotypes in England, 2007 to 2011. Data are stratified by quarter. (Reproduced from reference 213 with permission from Public Health England.)

designation can at times make interlaboratory exchange and epidemiological comparisons difficult. A limitation of both PFGE and ribotyping methods is that laboratories require a library of reference isolates for comparison in order to provide a "name" for strain types, and outside reference or public health laboratories, relatively few centers have access to such a library of strain types.

Multilocus variable-number tandem-repeat analysis. MLVA is emerging as a typing method for *C. difficile* to be used in outbreak investigations and appears to be discriminatory enough to monitor transmission events in the hospital setting (211, 215–217). This method uses multiple variable-number tandem-repeat (VNTR) loci, which are dispersed throughout the genome and vary with regard to level of diversity to resolve phylogenetic relationships between isolates. The amplicons that are obtained are resolved by using capillary electrophoresis, followed by automated analysis of the fragments. The "summed tandem-repeat difference" (STRD) is used as a measure of genetic difference between isolates. To date, a consensus approach toward MLVA typing for *C. difficile* has not been established. Various approaches to MLVA have been reported in the literature, each evaluating seven loci on the *C. difficile* genome (215, 216, 218). Manzoor and colleagues reported an approach which they term "extended MLVA" (eMLVA), which uses eight novel loci in addition to the seven loci previously reported (211). Using 229 previously ribotyped isolates from diverse locations around the world between 1996 and 2010, this group was able to efficiently subtype all of the isolates in the study while clustering like isolates concordant with PCR-ribotyping. The *D* value reported for this eMLVA method was 0.999, whereas PCR-ribotyping for the same group of isolates resulted in a *D* value of 0.886 (211). Wei et al. evaluated 142 *C. difficile* isolates by MLVA using different numbers of loci to devise a scheme with an adequate discriminatory index but with correlation to established PCR-ribotypes (219). Using various combinations of

VNTR loci, they devised MLVA schemes, each with discriminatory indices of 0.99, compared to PCR-ribotyping, which had a *D* value of 0.96 for the same subset of isolates. The reported *D* value for MLVA in the method comparison study by Killgore et al. was 0.964 (206).

MLVA can be useful for outbreak investigations and studies evaluating local transmission events, but as of yet, it remains nonstandardized; there is no established method or naming scheme that is portable across laboratories. MLVA is discriminatory and reproducible, and if agreement can be reached regarding the optimal method, the results could be portable and provide interlaboratory comparability.

Multilocus sequence typing. MLST is now commonly used on many pathogens to assess the general population structure and overall diversity within a species. MLST typically uses allele fragments that are 300 to 600 bp in length; the historical reason for this is that fragments of this size can be reliably and reproducibly read on a single sequencing run using Sanger sequencing methods. Most MLST schemes use between 6 and 12 loci, whereby each unique combination of alleles is assigned its own sequence type (ST), and databases of MLST data and STs are available in the public domain (220, 221). The loci used are typically housekeeping genes, as these are relatively conserved within a species, but variants within these housekeeping genes can be used to distinguish strains. Essentially, isolates are grouped based on their evolutionary proximity. When a novel allele is found, the investigator can apply to have it added to the ST database. This platform therefore allows for portability of results between different laboratories; on the Internet, freely accessible, curated databases of STs exist, making this method portable between laboratories (220).

C. difficile is theoretically well suited to MLST, as the species is relatively genetically heterogeneous. MLST was first described for *C. difficile* typing in 2004 by Lemee and colleagues, who used seven

housekeeping genes (*aroE*, *ddl*, *dutA*, *tpi*, *recA*, *gmk*, and *sodA*) to analyze a group of *C. difficile* isolates (222, 223). The *D* value for this method was calculated to be 0.958 (222). This was followed by a study by Griffiths et al., who performed MLST on 152 isolates of *C. difficile* isolated in culture, representing 49 ribotypes. The *D* value of the MLST method was 0.90, which was roughly comparable to the *D* value for PCR-ribotyping of the same collection, 0.92 (224). This group also applied this MLST typing scheme directly to stool specimens (rather than cultured isolates of *C. difficile*), with good success. For a small number of the stool specimens, sequencing failed as a result of inadequate amplification directly from the stool specimens. For the specimens where adequate amplification was obtained, the MLST data correlated with the ST of the isolate recovered in culture. The *D* value reported for MLST in the study by Killgore et al. was 0.699, which is lower than that reported by Lemee et al. and Griffiths et al. (207). This may reflect the fact that the study by Killgore et al. contained a larger proportion of NAP1/ribotype 027 strains and therefore inherently represented less diversity than the other two investigations (206, 222, 224).

Whole-genome sequencing. Whole-genome sequencing (WGS) has the potential to be the "ultimate" typing method, and the extra resolution can facilitate evaluation of specific transmission events within clusters or outbreaks (225, 226). Historically, WGS had been considered to be too expensive and cumbersome, with a turnaround time inadequate for real-time outbreak investigations. However, the cost is decreasing consistently, while the speed and throughput of WGS continue to improve (227–229). One challenge or limitation to WGS is still the analysis; adequate bioinformatics are required to make use of the enormous amounts of data that are generated.

Recently, WGS was used to study the transmission of *C. difficile* whereby WGS was performed on 486 samples from cases documented over 4 years in Oxfordshire, United Kingdom (230), using the Illumina platform. This analysis identified instances of direct transmission for a subset of the isolates, but direct transmission was ruled out for the majority of isolate pairs where direct transmission events would have been suggested by using conventional molecular typing techniques. In another pilot study, benchtop WGS was performed in almost real time to evaluate a *C. difficile* outbreak; samples from all cases identified over a 6-week period in one hospital were sequenced and compared with local strain sequences from the previous 3 years. Analysis of these strains illustrated that WGS could provide early outbreak detection and also suggested community transmission, which was not previously suspected (196).

The ribotype 027/NAP1 strains have emerged worldwide and are widespread in health care facilities. While the underlying reasons for this are postulated and described in previous sections of this paper, the reason for the rapid emergence and spread around the globe remain unknown. WGS also has a role in answering this question. He et al. used WGS for phylogenetic analysis and surprisingly found that two distinct lineages of this epidemic strain emerged in North America, termed FQR1 and FQR2 (45); they both acquired the same fluoroquinolone resistance-conferring mutation and a closely related transposon. The FQR2 lineage became more widespread, leading to outbreaks associated with health care institutions in Europe, the United Kingdom, and Australia (45).

WGS is reproducible and portable and does not require a library of established isolates for strain type determination. Although the cost and turnaround time continue to limit the routine use of WGS at the time of preparation of the manuscript, it is likely that WGS will be more widely adopted for *C. difficile* strain typing in the future.

TESTING CONSIDERATIONS FOR THE CLINICAL MICROBIOLOGY LABORATORY

Several professional societies and other organizations have published guidelines for the diagnosis and treatment of *C. difficile* disease (52, 101, 231, 232). These organizations include the SHEA/IDSA in the United States (52), the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (101), and the Australian Society for Infectious Diseases (232). All of the guidelines have a list of patients for whom a diagnosis of *C. difficile* disease should be considered; they also recommend that any hospitalized patient who has ≥3 unformed stools in a 24-h period should be tested. It is recommended that testing be performed only on unformed stool except in rare instances, such as an ileus (232). Laboratories should have specimen rejection policies that restrict testing to watery, liquid, or unformed stools that take the shape of the container. Asymptomatic patients should not be tested, even for test of cure.

Repeat testing is of limited value but is still somewhat controversial. The practice of sending "stools for *C. difficile* times three" became the established norm in many clinical laboratories after the widespread implementation of insensitive enzyme immunoassay methods (233) (the reader is referred to the section above on EIA testing for more details). Physicians would send multiple specimens to the laboratory to compensate for the poor performance of these tests. Numerous studies have demonstrated that regardless of the testing method, EIA (149, 234–236), cell culture cytotoxicity neutralization assays (237, 238), or NAATs (149, 239, 240), and the patient population tested, sending of repeat samples does not increase the yield and can be misleading, as the positive predictive value drops with each subsequent test (149). One systematic review of the literature noted that the yield with a second EIA after an initial negative result dropped to 1.5 to 4.7% in hospitalized patients (241). In an outbreak situation, this review and other studies noted that the yield of repeat testing may be higher (5%), suggesting that outbreak situations may be the one situation where repeat testing may be helpful (234, 241). At least one study has demonstrated that substantial cost savings may be incurred by eliminating duplicate testing without adversely affecting patient care (239). Since longitudinal studies suggest that after 7 days, the frequency of positive results begins to increase again, most likely reflecting new infections as opposed to false-negative tests (101, 234), many laboratories have implemented a 7-day rule; that is, repeat tests following an initial negative result will not be performed within a 7-day period of time.

SPECIAL PATIENT POPULATIONS

Children

High rates of *C. difficile* colonization have been well documented in infants less than 1 year of age (242–245). Infants who are breast-fed appear to have lower carriage rates (14%) than formula-fed infants (30%) (246). One study illustrated that infants were usually colonized with a single clone of *C. difficile* and that they were colonized for several consecutive months (242). Once new foods are introduced to infants, they may acquire new strain types of *C. difficile* (242).

Infants are almost invariably asymptomatic, although it can be

difficult to discriminate asymptomatic carriage from true disease in infants <1 year of age. The mechanism and pathobiology of this asymptomatic colonization in neonates are unknown, but many hypotheses, including passive transfer of protective maternal antibody and a protective fecal microbiome in infancy, have been offered. There is some thought that infants lack the receptor for the toxin, but considering that the receptor for toxin B is as yet uncharacterized, this remains unproven.

Both nosocomial and community-associated *C. difficile* infections are on the rise in the pediatric population (247–250). Between 2001 and 2006, one study reported an increase in the incidence of *C. difficile* disease in children from 2.6 to 4.0 cases per 1,000 hospitalized children (247); similarly, another study evaluated *C. difficile* in hospitalized pediatric patients and found that from 1997 to 2006, the incidence of *C. difficile* disease was 35 per 10,000 hospitalizations in children 5 to 9 years of age (249). NAP1 strains have been recovered from pediatric patients (251, 252).

Many pediatric institutions are converting or are in the process of converting to molecular methods for detection of *C. difficile* infection. When molecular testing is used on pediatric samples, the increased sensitivity leads to an apparent increase in the incidence of *C. difficile* disease in pediatric patients, just like adults. Few studies have evaluated the performance characteristics of various diagnostic assays for pediatric patients specifically. One of the first studies using a NAAT for pediatric patients was conducted by Luna et al., who tested 157 samples from 96 pediatric patients; toxigenic culture was the gold standard in this investigation. The laboratory-developed PCR assay used in this study was found to be 95% sensitive (253), and once the PCR assay was implemented (2007 to 2010), the overall volume of testing remained constant, but the *C. difficile* positivity rate nearly doubled compared to when the TechLab Tox A/B II EIA was used (2003 to 2006) (253).

Selvaraju et al. examined 200 stool samples from pediatric patients using toxigenic culture as the gold standard. Those researchers evaluated the C. Diff Quik Check Complete, BD GeneOhm, and ProGastro Cd PCR assays. The sensitivity and specificity of each of these assays were as follows: 97.9 and 82.2% for C. Diff Quik Chek Complete, 89.6 and 96.7% for GeneOhm PCR, and 100 and 93.4% for ProGastro PCR, respectively (122). In this study, a retrospective chart review was performed for all patients who were positive for *C. difficile* by PCR methods only; for 3 of these patients, *C. difficile* was a plausible diagnosis, but for the remaining 8 patients, the clinical picture was not compatible with a diagnosis of *C. difficile*. The authors of this study concluded that future prospective studies of pediatric patients are needed to fully elucidate the clinical significance of a positive NAAT assay for *C. difficile* as well as the economic impact of NAAT testing in this patient population (122).

A study by Ota and McGowan evaluated GDH, toxin EIA, and NAAT assays, alone and as part of multistep algorithmic approaches. This was a prospective study including 141 samples from patients aged 1 to 18 years (64). Similar to the findings of Selvaraju et al., and similar to assay evaluations of adult patients, the conclusions of this study were that toxin EIA had the lowest sensitivity for *C. difficile* detection (56%) and that a molecular assay (the Illumigene *C. difficile* assay) exhibited increased sensitivity (89%), although the sensitivity of this assay in this population is somewhat lower than what has been reported in other studies (64). This study does not reveal an assay that is clearly superior for the diagnosis of *C. difficile* infection in pediatric patients.

Although molecular methods may be problematic for *C. difficile* diagnosis in pediatric patients, toxin EIAs may also produce false-positive results. Toltzis and colleagues performed toxigenic culture on 112 stool specimens from pediatric patients that had previously tested positive for *C. difficile* by the Meridian Premier Toxin A & B assay (254). Those authors found that toxigenic *C. difficile* could be isolated from only 72 of these specimens. The average age of the children with EIA-positive, culture-negative samples was significantly lower than that of the children with EIA-positive, culture-positive samples (0.53 years versus 2.86 years; $P = 0.002$), but otherwise, no differences between the two groups of patients were detected (254). Thus, the PPV of a positive EIA for *C. difficile* in pediatric patients is also suboptimal, which is thought to be related to the low pretest probability for disease in the patients. No methodology is clearly superior for making the diagnosis of *C. difficile* disease in this patient population.

It should be noted that not all of the FDA-cleared *C. difficile* assays have been evaluated or are approved for testing of specimens from patients <2 years of age. It is incumbent upon laboratories performing these tests to confirm the indications for their assay and perform additional validation and verification if appropriate.

The American Academy of Pediatrics has recently released a policy statement on *C. difficile* infection in infants and children (255). The guidelines caution that testing for *C. difficile* should be performed only for children who meet the criteria for clinically significant diarrhea and that test results for infants <1 year of age can be difficult to interpret due to high rates of asymptomatic colonization. Some of the general recommendations of this document are that for children >3 years of age, diagnostic testing can be performed as it would be for adult patients. In children 1 to 3 years of age with diarrhea, causes other than *C. difficile* (such as viral causes) should be considered and tested for in addition to *C. difficile* when *C. difficile* is suspected. The guidelines also caution that no diagnostic test should be used as a test of cure, just as is the case for adult patients.

Oncology Patients (Nontransplant)

Risk factors for *C. difficile* infections among oncology patients include chemotherapeutic agents; antibiotics, especially cephalosporins and clindamycin; frequent hospitalizations; and prolonged neutropenia (256–258). While it might be anticipated that cancer patients receiving chemotherapy will have a severe course and increased risk of death compared to other hospitalized patients, in at least one study, this was not the case (259). In a study by Stewart et al., patients with hematological malignancy and *C. difficile* disease had zero colectomies to control disease, compared to four colectomies in the group without malignancy (259). No differences were noted in terms of other markers of disease severity, such as the need for intensive care unit admission (259). The only difference noted was a longer length of stay for patients with *C. difficile* colitis and malignancy (259). This study was limited by the small numbers of patients.

Solid-Organ Transplant Recipients

Longitudinal studies have shown a dramatic increase in the incidence of *C. difficile* among solid-organ transplant (SOT) recipients that parallels that of the disease in the general population and other at-risk groups. In a study by Boutros et al., the incidence of *C. difficile* disease was 4.5% in 1999, peaked at 21.1% in 2005, and plateaued at 9.5% in 2010 (260). These authors also noted that signifi-

cant independent risk factors for *C. difficile* disease included a transplant other than kidney, age of >55 years, and induction with antithymocyte globulin (260). Once a patient with a SOT develops *C. difficile* disease, there is an independent association with greater mortality; longer length of stay, with the associated increased costs of hospitalization; more complications associated with the transplanted organ; and an increased need for colectomy (261).

Hematopoietic Stem Cell Transplant Patients

Diarrhea is common among hematopoietic stem cell transplant (HSCT) recipients, but until recently, *C. difficile* was relatively uncommon (262). A recent review reports the incidence of *C. difficile* disease among HSCT patients to be as high as 20% (263). In most studies, there do not appear to be any unique risk factors among HSCT recipients that are not shared by other nontransplant at-risk patients (256, 263). However, in a large study by Dubberke et al., patients who received a third- or fourth-generation cephalosporin were more at risk for development of *C. difficile* disease (264). This study also demonstrated that the receipt of growth factors was associated with decreased risk (264). These findings, especially the potentially protective effect of growth factors, need to be verified by prospective studies.

Once patients develop symptomatic *C. difficile* disease, the incidence of graft-versus-host disease (GVHD) greater than grade 2 and mortality not related to relapse of the underlying malignancy are higher than in uninfected patients (264, 265). In a study by Alonso et al., there was a strong relationship between early *C. difficile* disease and the development of GVHD in the year following infection. More prospective studies assessing the relationship between *C. difficile* and GVHD are needed (266).

Patients with Inflammatory Bowel Disease

As is the trend among the general population, studies have shown that the frequency of *C. difficile* disease among patients with inflammatory bowel disease (IBD) has increased by 2- to 3-fold since 2000 (267–269). In contrast to what is observed for the non-IBD population, two studies demonstrated that 66 to 75% of IBD patients acquired infection in the outpatient setting and that only 61% had recent antibiotic exposure (267, 268). Risk factors for *C. difficile* infections in IBD patients from these and other studies include increasing age, comorbidities, malignancies, and immunomodulatory therapy (267, 268). Immunosuppression increases the risk 2-fold (270). Several studies reported an increased risk of disease in patients with ulcerative colitis (UC) (colonic disease) compared with those with Crohn's disease as well as a greater risk with pancolitis than with distal disease (267, 270, 271). These observations have resulted in the recommendation that any IBD patient with symptoms severe enough to result in hospitalization should be tested for *C. difficile* upon admission, even in the absence of traditional risk factors such as antibiotic exposure (269, 271). Since there are no distinguishing clinical features that discriminate a flare of inflammatory bowel disease from diarrhea associated with *C. difficile*, testing is warranted for any IBD patient with significant diarrhea.

Many studies also demonstrated that *C. difficile* can contribute substantially to increased morbidity and mortality among IBD patients compared to non-IBD controls (270, 272). Mortality, especially among patients with UC; the need for bowel surgery; and duration of hospitalization are all substantially increased (267, 270, 272).

While there appears to be a substantial impact on the course of IBD in patients with active *C. difficile* disease, more definitive literature is needed to prove that *C. difficile* actually causes a flare of IBD. Likewise, there are no data to support that the various testing methods for *C. difficile* diagnosis perform differently among these patients than among controls. That said, as with non-IBD patients, patients with IBD can be asymptomatically colonized with *C. difficile*, and therefore, the same criteria for testing, namely, significant diarrhea, should be applied.

SUMMARY AND CONCLUSIONS

C. difficile continues to evolve, and the changing biology and growing lists of patient groups at risk have resulted in an unprecedented surge in the incidence and burden of disease. This review details the methods for diagnostic testing for *C. difficile*. New strain types have emerged, which have led to a marked increase in the incidence of *C. difficile* disease since the early 2000s. The increased incidence of *C. difficile* disease has highlighted the lack of sensitivity of the toxin EIAs that are used by most laboratories for the diagnosis of *C. difficile* and has ushered in a new era of molecular assays for diagnosis. At present, eight FDA-approved molecular assays for *C. difficile* are on the market. However, these assays do not solve the diagnostic uncertainty surrounding *C. difficile*, as detection of *C. difficile* in a fecal specimen does not automatically imply disease. *C. difficile*-associated disease is a clinical diagnosis supported by laboratory findings; this diagnosis continues to be a challenge for clinicians and laboratories alike.

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