nique can be used to identify HIV-1-infected children. The commercial assay represents a considerable advantage over the more labor intensive and inconvenient radioisotopic PCR methods. This test is simple to perform with standard equipment requirements and results are available within five hours. Also important is the fact that the use of assays that provide standardized reagents and procedures for detection of HIV-1 DNA sequences would significantly reduce interlaboratory variability.

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Role of Culture and Toxin Detection in Laboratory Testing for Diagnosis of *Clostridium difficile*-Associated Diarrhea

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Two variations of an egg yolk agar base medium containing cycloserine, cefoxitin, and fructose (CCFA), one with 250 μg and the other with 500 μg of cycloserine/ml of agar medium were compared

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to study the effect of the cycloserine concentration on recovery of *Clostridium difficile* from stool samples. In addition, the role of prior anaerobic reduction of these media in the detection of *Clostridium difficile*-associated diarrhea (CDAD) was tested. Each medium was studied over a two-month period, with outcome compared between the testing periods and to historical data from our institution. Clinical correlation of test results was performed. The use of the originally described formulation of CCFA with 500 μg of cycloserine/ml of agar combined with 4 h of anaerobic reduction prior to specimen inoculation increased the rate of isolation of toxigenic *Clostridium difficile* from clinical specimens from 6 to 17% ($p < 0.001$). Combining direct detection of stool toxin and properly performed culture for toxigenic *Clostridium difficile* enhances the potential for diagnosis of CDAD. For optimal performance the culture medium should contain the originally proposed cycloserine concentration of 500 μg/ml of agar and should be anaerobically reduced at least 4 h prior to specimen inoculation.

Clostridium difficile-associated diarrhea (CDAD) is a widespread, primarily nosocomial infection. A recent review by Kelly et al. (1) highlights the important features of this disease, from diagnosis to therapy. However, the subsequent letter by Fang and Madinger (2) and the original authors' reply (3) indicate persisting misunderstandings as to the role of culture in the diagnosis of this infectious disease. In our recent review of the role of the clinical microbiology laboratory in the management of CDAD, we found culture to be of critical importance for both the diagnosis and the epidemiology of this disease (4). We cited data indicating that while direct detection of toxin in stool is highly specific, the sensitivity of toxin detection when used alone approaches only 70%, thus implying that up to 30% of patients whose clinical findings support the diagnosis of CDAD will be missed. Culture, appropriately performed, can provide laboratory confirmation of a clinical diagnosis (4). With the recent emergence and spread of vancomycin resistance in gram-positive organisms, including the demonstration that enterococcal resistance to vancomycin can be transferred into clostridia via plasmids (5), the detection of *Clostridium difficile* in culture may become even more important. The determination of precise epidemiology for development of appropriate infection control practices also requires the recovery and collection of viable organisms to efficiently determine the clonal spread of infection.

Our review highlighted the requirements for optimal detection in culture of toxigenic *Clostridium difficile* from potentially infected patients (4), based on work such as that reported by Mundy et al. (6). An apparent discrepancy exists over the role of culture as evidenced by differences between our review (4) and the comments of Kelly et al. (1, 3). This combined with historical data from the laboratory at Northwestern Memorial Hospital demonstrating poor recovery of *Clostridium difficile* from symptomatic patients compared to toxin B detection in cell culture, prompted us to undertake a two-part evaluation of medium factors that influence the sensitivity of culture for detection of toxigenic *Clostridium difficile* from the stool of patients at risk for CDAD. In this study we specifically evaluated the effect of prior anaerobic reduction of the medium used for culture and the effect of the cycloserine concentration on recovery of *Clostridium difficile* from stool samples.

Materials and Methods. Each stool specimen received for *Clostridium difficile* testing was prepared for both culture and cytotoxin assay. The culture was inoculated in duplicate for the two media investigations. Commercial sources were chosen to supply the medium so that the results would be more directly applicable to routine clinical microbiology laboratories. Plates were utilized for culture within three weeks of medium preparation. All media purchased passed in-house quality control testing, which requires that isolated colonies of American Type Culture Collection (ATCC, Rockville, USA) *Clostridium difficile* strains 9689 and 17858 grow to at least 3 mm diameter in 48 h at 35°C when plated on anaerobically reduced medium. The strains must produce flat, yellow, ground-glass appearing colonies with a surrounding yellow halo in the medium. The Gram stain of these colonies must show typical morphology, and the medium must inhibit the growth of *Escherichia coli* ATCC 25922.

In test period 1 of the study (November and December 1993), one portion of the specimen was tested by dipping a sterile swab into the unformed stool and plating it onto a nonreduced egg yolk agar base medium containing cycloserine, cefoxitin, and fructose (CCFA). This *Clostridium difficile* agar plate (cat. no. 21852, BBL, Becton Dickinson Microbiology Systems, USA) containing 250 μg cycloserine/ml agar medium was inoculated within 2 h of specimen receipt in the laboratory. Within 15 min of inoculation, this plate was incubated at 35°C in a double-valve anaerobic jar that had been vacuumed and flushed three times

Table 1: Comparison of media and incubation conditions prior to, during, and following the various *Clostridium difficile* culture study periods.

Period	Cycloserine concentration	Prior anaerobic reduction	Total stools tested (average/month)	No. (%) of stools with toxigenic *C. difficile*	No. (%) of stools with cytotoxin
Jan.–Sept. 1993	250 µg/ml	no	1509 (168)	90 (6.0%)[a]	136 (9.0%)
Nov.–Dec. 1993	250 µg/ml	no	240 (120)	14 (5.8%)	16 (6.7%)
		yes	240 (120)	17 (7.1%)[b]	16 (6.7%)
May–June 1994	500 µg/ml	no	335 (168)	41 (12.2%)[a,d]	29 (8.7%)
		yes	335 (168)	52 (15.5%)[c,d]	29 (8.7%)
July–Oct. 1994	500 µg/ml	yes	772 (193)	108 (14.0%)[c]	62 (8.0%)

[a]Difference from stool cytotoxin significant, $0.001 < p < 0.01$.
[b]Difference from stool cytotoxin not significant, $p > 0.2$.
[c]Difference from stool cytotoxin significant, $p < 0.001$.
[d]Difference between media significant, $0.001 < p < 0.01$.

with a mixture of anaerobic gas (85% nitrogen, 10% hydrogen, 5% carbon dioxide). A second portion of the specimen was inoculated onto another BBL *Clostridium difficile* agar plate (same manufacturer's lot) that had been reduced for a minimum of 4 h in an anaerobic chamber (Bactron Anaerobic/Environmental Chamber, Sheldon Manufacturing, USA) containing 90% nitrogen, 5% hydrogen, and 5% carbon dioxide. After inoculation the plate was placed into an anaerobic jar. This second set of cultures was prepared daily as a single batch from specimens held less than 24 h at 4–8°C.

All plates were first removed from the anaerobic jars at 48 h and examined for growth of flat or umbonate colonies 2–9 mm in diameter with a filamentous edge, having a ground-glass appearance and changing the color of the surrounding medium yellow. Colonies fulfilling these characteristics were further evaluated for identification as *Clostridium difficile*. Those isolates appearing as gram-positive bacilli on Gram stain, not growing aerobically, and possessing a characteristic odor were considered to be *Clostridium difficile*. Any anaerobic organisms from colonies described above but without all these characteristics were analyzed by gas-liquid chromatography for the characteristic pattern of *Clostridium difficile*. Negative plates were incubated and examined in the anaerobic chamber for an additional five days at 35°C before final results were reported as positive or negative for *Clostridium difficile*.

Table 2: Results of the *Clostridium difficile* medium test during May and June 1994 (period 2) compared to clinical findings in potentially infected patients.

Category	No. (%)			
	Stool toxin positive, toxigenic culture positive	Stool toxin negative, toxigenic culture positive	Stool toxin positive, culture negative	Stool toxin negative nontoxigenic culture positive
Stool specimens (n = 63)				
Diarrhea				
With Antimicrobial therapy	16 (25)	18 (29)	2 (3)	7 (11)
Without Antimicrobial therapy	1 (2)	0 (0)	0 (0)	1 (2)
No Diarrhea				
With Antimicrobial therapy	9 (14)	6 (10)	0 (0)	1 (2)
Without Antimicrobial therapy	1 (2)	1 (2)	0 (0)	0 (0)
Patients (n = 42)[a]				
Diarrhea				
With Antimicrobial therapy	12 (29)	13 (31)	2 (5)	5 (12)
Without Antimicrobial therapy	0 (0)	0 (0)	0 (0)	1 (2)
No Diarrhea				
With Antimicrobial therapy	6 (14)	7 (17)	0 (0)	1 (2)
Without Antimicrobial therapy	1 (2)	0 (0)	0 (0)	0 (0)

[a]Some patients are listed more than once due to different results obtained with separate specimens.

Growth was graded semiquantitatively as 1+ to 4+, depending on how many quadrants colonies of *Clostridium difficile* were found growing.

Test period 2 of the study (May and June 1994) differed from the first period in only one component of the medium formulation evaluated. Here we tested CCFA agar (cat. no. 60-0461, Dimed, USA) using a cycloserine concentration of 500 μg/ml of agar. The other components of the medium were unchanged. This second study period used the same cycloserine formulation suggested by Mundy et al. (6), which is based on the original formulation of George et al. (7).

Cytotoxin testing was performed in tissue culture using MRC-5 cells. Stool samples were mixed with an equal volume of phosphate-buffered saline and centrifuged to remove debris. The supernatant was then filtered through a 0.45 micron filter. The specimen was tested in the tissue culture tube at a final dilution of 1:200 (4). Cells were examined for cytotoxicity at 24 and 48 h, and any sample showing cytotoxicity was neutralized subsequently with antitoxin. A stool specimen demonstrating cytotoxicity that is neutralized by antitoxin directed against *Clostridium difficile* cytotoxin is considered positive for toxin B (4). Toxin testing of *Clostridium difficile* strains was performed by inoculating an isolated colony into prereduced chopped meat broth and incubating anaerobically at 35°C for two days. Detection of cytotoxin in the broth supernatant was performed in the same manner as testing of stool specimens.

Only unformed stools were accepted for processing. All others were rejected. The available medical records of patients with any positive test results were reviewed for the likelihood of disease consistent with CDAD during the May and June 1994 (period 2) evaluation of CCFA, which contained cycloserine at a concentration of 500 μg/ml. The pattern of the positive test results was unknown to the reviewer. Criteria necessary for the clinical diagnosis of CDAD were the same as in prior studies and included the following: i) passing of at least three stools per day for at least two days; ii) antimicrobial therapy received within the past two months; iii) no other obvious reason for diarrhea; and iv) treatment for CDAD administered by an attending physician.

In addition to comparing the results of the two study periods to each other, we compared the results of each of them to the results of routine clinical laboratory testing performed over the nine months preceding period 1 (November–December 1993) and the four months following period 2 (May–June 1994). In the nine months prior to period 1, CCFA medium with 250 μg/ml cycloserine was used, without prior anaerobic reduction. In the four months following period 2, CCFA containing 500 μg/ml cycloserine was used, with the medium being anaerobically reduced for ≥ 4 hours prior to use. Statistical analysis was performed using the chi-square test.

Results and Discussion. The data from our testing in the various time periods, including the results correlated with the clinical investigation in May and June, are shown in Tables 1 and 2. Table 1 shows a comparison of the media used during the two study periods and including the results of cytotoxin testing as well as the time periods preceding and following these investigations. It clearly shows that the higher, original cycloserine formulation enhances the detection and subsequent recovery of *Clostridium difficile* and that prior anaerobic reduction of the medium is especially beneficial when the 500 μg/ml cycloserine concentration is used. Table 2 presents laboratory test results along with the clinical char review data, documenting the association between clinical findings and the culture-plus-cytotoxin test result patterns for CDAD during May–June 1994.

A detailed comparison of the in vitro test data evaluating the effect of cycloserine content and prior anaerobic reduction on medium performance during test periods 1 and 2 supports these results. The group of 240 samples from period 1 using 250 μg/ml cycloserine contained 16 samples that were positive for both stool cytotoxin and growth of toxigenic *Clostridium difficile*. Two of these grew only on prior-reduced medium, four had more colonies on the prior-reduced medium, four had similar numbers on both the prior-reduced and nonreduced medium, and six had less growth on the prior-reduced medium. In this study only one cytotoxin-negative stool sample was positive for toxigenic *Clostridium difficile* in culture, and that was on the prior-reduced medium. Nontoxigenic *Clostridium difficile* was recovered from two samples, on both the prior-reduced and the non-reduced medium. Markedly different results were found for the 335 samples from period 2 cultured on medium containing 500 μg/ml cycloserine. Thirty-one samples were positive in both the stool cytotoxin test and culture of toxigenic *Clostridium difficile*: three grew only on the prior-reduced medium, 17 grew more colonies on the prior-reduced medium, eight grew similar numbers on both the prior-reduced and the nonreduced media, and three had less growth on

the prior-reduced medium. In this second study (period 2) 26 cytotoxin-negative stool samples were positive for toxigenic *Clostridium difficile* in culture: eight were positive only on the prior-reduced medium, nine grew more colonies on the prior-reduced medium, and nine exhibited similar growth on both the prior-reduced and the nonreduced media. No additional positive samples were found only on the nonreduced CCFA. Eleven isolates of nontoxigenic *Clostridium difficile* were recovered: four were detected only on the prior-reduced medium, two grew more colonies on the prior-reduced medium, and five exhibited similar growth on both the prior-reduced and the nonreduced media. The data demonstrate that prior anaerobic reduction of the medium not only facilitates detection of more *Clostridium difficile* but also enhances the growth of the organism compared to the nonreduced medium. This effect is suggested by growth obtained at the lower cycloserine concentration and is well demonstrated in the medium containing the higher concentration (500 μg/ml) of cycloserine.

Prior to starting our investigation (January through September 1993; Table 1), culture for *Clostridium difficile* detected significantly fewer specimens positive for toxigenic strains than did direct cytotoxin testing of these same stools ($0.001 < p < 0.01$). During the November and December 1993 investigation (period 1), we found that 250 μg/ml cycloserine combined with prior anaerobic reduction only improved the performance of culture so that cytotoxin testing was no longer superior to culture ($p > 0.2$), demonstrating that prior anaerobic reduction had only a minor influence on improving performance in the absence of a higher cycloserine concentration. In these two months the nonreduced medium performed as it had in the past. In May and June 1994 (period 2), when 500 μg/ml cycloserine combined with prior anaerobic reduction was evaluated in 335 samples, the rate of cultures positive for toxigenic *Clostridium difficile* was 17% and that of cultures positive for nontoxigenic *Clostridium difficile* 3.3%, constituting the best test performance. Culture on both the reduced and the nonreduced media containing the higher cycloserine concentration was superior to direct stool cytotoxin testing (Table 2). July through October 1994 was a follow-up period, when all clinical testing was performed using the new culture procedure of prior anaerobic reduction of CCFA medium containing 500 μg/ml cycloserine. As can be seen from Table 1, the percent recovery of toxigenic *Clostridium difficile* (14%) was consistent with the other study period in which this approach was evaluated.

We were able to review the records of patients accounting for 63 of 72 positive test results (88%), representing 42 patients, during period 2. The data were evaluated based upon total tests and individual patient results (Table 2). The fact that the results of testing were similar for all patient symptom groups based on review of clinical records indicates that specimens positive for toxigenic *Clostridium difficile* in culture only (negative direct stool cytotoxin test) were as likely to be from patients with disease consistent with CDAD as those whose direct stool toxin test results was also positive.

When only the first result from each of these patients was evaluated, a similar outcome was found. In this analysis 35 patients were positive by either the stool cytotoxin test or culture for toxigenic *Clostridium difficile*, and of these, 25 had clinical criteria consistent with CDAD. There were 11 patients (31%) with diarrhea who were stool toxin negative and toxigenic culture positive and had received antimicrobial agents, five patients (14%) without significant diarrheal stools (not fulfilling our CDAD definition) who were stool toxin negative and toxigenic culture positive and had received antimicrobial agents, 12 patients (34%) with diarrhea who were stool toxin positive and toxigenic culture positive and had received antimicrobial agents, six patients (17%) without diarrhea who were stool toxin positive and toxigenic culture positive and had received antimicrobial agents, two patients (6%) with diarrhea who were stool toxin positive and toxigenic culture negative and had received antimicrobial agents, and one patient (3%) without diarrhea who was stool toxin positive and toxigenic culture positive and had not received antimicrobial agents. There were eight patients in whom multiple tests (range 2 to 7 samples) were performed, of which at least one test was positive. Initially, discordant results were found for five patients: all were culture-positive for a toxigenic strain of *Clostridium difficile* but were negative in the direct stool toxin test. Three became negative in both tests upon subsequent testing, one continued to have discordant results for the first three samples but became positive in both tests on samples 4 and 5, and one had discordant results for two replicate stool samples. Overall, when evaluating the data on the first specimen received from all the patients during the two-month evaluation, culture for toxigenic *Clostridium difficile* increased the

yield of laboratory confirmation of CDAD disease 78% by identifying 11 of the 25 positive patients that would have been missed based upon direct detection of stool cytotoxin alone.

Our evaluation confirms earlier data indicating that testing to detect toxin alone will result in a significant number (≥ 30%) of likely CDAD cases being missed (8, 9). This may reflect a previous unrecognized insensitivity of MRC-5 cells, but we believe more likely it reflects the enhanced recovery of *Clostridium difficile* when using the originally described cycloserine concentration along with prior anaerobic reduction of the media. We have routinely used a dilution cut-off of 1:200 to indicate a positive result in the cytotoxin test. We believe this provides a consistent degree of sensitivity and avoids a high rate of nonspecific cell toxicity. While some laboratories may choose to test at a lower specimen dilution, Walker et al. (10) demonstrated that lower stool dilutions (of approximately 1:20) are associated with increasing problems with nonspecific cytotoxicity. At this lower specimen dilution they found the tube cytotoxicity test to have a specificity of only 79% when diagnosis of CDAD included clinical criteria as well (10). Improved specificity was seen in microtiter wells at a dilution of 1:100, although false-positive results were not eliminated unless a final dilution of 1:1000 was considered as a cut-off for test positivity.

Barbut et al. (11) found that enzyme immunoassays were not sufficient for diagnosis when compared to either the traditional cytotoxicity assay or culture for detection of toxigenic *Clostridium difficile* strains. Finally, Bond et al. (12) recently reported a study of 500 stool specimens in which the utility of culture and stool toxin detection was evaluated. They detected stool toxin in 41 specimens from 32 patients. An additional four patients (12.5%) having disease compatible with *Clostridium difficile* infection were detected only by isolation of toxigenic *Clostridium difficile* strains (12). However, it is apparent that cultures must be performed carefully, using the procedure outlined in this report, and that laboratories as well as clinicians must insist on relevant patient data to confirm the diagnosis of *Clostridium difficile*-associated diarrhea and colitis. Willey and Bartlett (13) initially evaluated selective media containing 250 μg/ml cycloserine and found it to perform well in 18 selected fecal samples from 73 patients whose stools were known to be positive for cytotoxin. However, they did not evaluate the lower cycloserine concentration in a population of patients for whom the diagnosis of *Clostridium difficile*-associated diarrhea or colitis was in doubt.

Tables 1 and 2 show that it is important to use the original cycloserine concentration proposed by George et al. (7) and to anaerobically reduce the medium at least 4 h prior to inoculating clinical specimens for culture (6). We also found that commercial media meeting adequate quality control standards as outlined in the Methods are sufficient for good recovery of *Clostridium difficile*, making it possible for most clinical laboratories to perform this testing. Several years ago our laboratory had changed to a medium containing only 250 μg/ml cycloserine because of reports that the 500 μg/ml formulation could be inhibitory to some *Clostridium difficile* strains (14). However, in the present study we found that the lower concentration of cycloserine permits many plates to be interpreted as negative for *Clostridium difficile* because of overgrowth with other stool flora.

Clostridium difficile-associated diarrhea causes significant morbidity, and even mortality, in institutionalized patients. Sensitive tests in the clinical microbiology laboratory are mandatory for both detection and eventual control of this disease through understanding the precise epidemiology of this infection (15, 16). Furthermore, in the case of therapeutic failure with vancomycin, culture would be crucial for organism recovery in order to document continued susceptibility of *Clostridium difficile* to the antimicrobial agent selected for treatment. Therefore, we disagree with Kelly et al. (1, 3) that current technology permits the cytotoxin assay to be an adequate gold standard for the laboratory confirmation of CDAD, since it is simply not sufficiently sensitive when culture for *Clostridium difficile* is performed properly. It is our continued belief that current methodology necessitates both testing for toxin (either A or B) and culturing for toxigenic *Clostridium difficile* to achieve this goal. Culture is needed not only for epidemiological evaluation but also for primary diagnosis of CDAD.

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Comparison of an Automated Enzyme Immunoassay with a Direct Fluorescent Antibody Test and Polymerase Chain Reaction for the Detection of *Chlamydia trachomatis* in Diagnostic Specimens from Male Patients

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Endourethral swabs and first-pass urine (FPU) samples from 148 male patients were tested for *Chlamydia trachomatis* by an automated enzyme immunoassay (EIA) (Vidas; bioMérieux, France), a direct fluorescent antibody (DFA) test (MicroTrak; Syva, USA) and two polymerase chain reaction (PCR) methods. *Chlamydia trachomatis* was considered present if a specimen was positive by at least two methods. This expanded criterion identified 27 patients (18%) as truly infected. One of the PCR methods was most sensitive for both types of specimen. When the recommended cut-off value of Vidas was reduced by 50%, its sensitivity on endourethral swabs was comparable to that of the DFA test, but the DFA test performed better with FPU. In general, FPU was suitable only for PCR.

Chlamydia trachomatis is an important cause of sexually transmitted disease. For many years cell culture was the gold standard in the laboratory diagnosis of *Chlamydia trachomatis* infection (1). However, it is increasingly evident that some of the newer molecular nonculture methods are more sensitive than cell culture (2–5). As a result, many researchers have tried to introduce expanded gold standards in the evaluation of tests for *Chlamydia trachomatis* (2, 3, 5). Here, we describe a prospective comparison of three different

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