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Concurrence of *Clostridium difficile* Toxin A Enzyme-Linked Immunosorbent Assay, Fecal Lactoferrin Assay, and Clinical Criteria with *C. difficile* Cytotoxin Titer in Two Patient Cohorts

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The accurate and sensitive diagnosis of *Clostridium difficile*-related diarrhea, normally treated with vancomycin, has become increasingly important in light of the emergence of dangerous new strains of vancomycin-resistant enterococci. In order to improve the threshold for *C. difficile* diagnosis and treatment, a number of commonly used assays for the diagnosis of *C. difficile* diarrhea were examined. These included an enzyme-linked immunosorbent assay for *C. difficile* toxin A (ToxA), a CHO cell culture assay for fecal *C. difficile* (cyto)toxin B, and a lactoferrin latex agglutination assay for fecal lactoferrin (LFLA). We studied 722 fecal specimens submitted by physicians for *C. difficile* toxin testing at the Salem, Va., Veterans' Affairs Hospital and at the University of Virginia Medical Center in Charlottesville. Charts were reviewed from 123 Veterans' Hospital patients and 114 University of Virginia patients for clinical criteria indicative of *C. difficile* diarrhea. An increasing titer of CHO cell cytotoxicity was correlated with an increasing likelihood of ToxA positivity (5 to 90%), LFLA positivity (39 to 77%), and clinical agreement (28 to 85%). However, some data indicate that the CHO cell cytotoxicity assay may be nonspecific when positive only at low titers. When the CHO assay result is positive at high titers, it remains the best diagnostic tool. Yet, when it is positive at a low titer, careful interpretation of the results in conjunction with other assays and the clinical setting is warranted, especially in light of new drug-resistant strains of microorganisms.

Clostridium difficile, a gram-positive, anaerobic, spore-forming bacterium, has long been recognized as a major cause of nosocomial diarrhea and pseudomembranous colitis in persons receiving antibiotic therapy. *C. difficile*-associated diarrhea results when antimicrobial agents alter the normal colonic flora and allow *C. difficile* to overgrow in the colon. Toxigenic strains of *C. difficile* produce two toxins: toxin A (an enterotoxin) and toxin B (a cytotoxin). It appears from animal models that toxin A is primarily responsible for the disease (10, 18). *C. difficile* colitis is easily treated, usually with oral metronidazole or vancomycin (2, 10). However, neither of these agents will eradicate *C. difficile* (9) and their use is associated with the dangerous emergence of enterococci, including vancomycin-resistant enterococci (5). Consequently, the need for accurate, sensitive means to select those patients with *C. difficile* colitis in need of specific antimicrobial therapy has grown increasingly important.

A number of testing methods exist for laboratory diagnosis. An enzyme-linked immunosorbent assay (ELISA) for toxin A is commonly used with high specificity (11). In addition, a latex bead agglutination test for fecal lactoferrin (concentrated in the granules of polymorphonuclear leukocytes) provides an additional measure of inflammatory enteritis (7) that is more sensitive in *C. difficile* toxin-positive specimens (75%) than is direct methylene blue microscopy for fecal leukocytes (40%) (20).

The most valued test for *C. difficile* evolved in the late 1970s after several investigators demonstrated that *Clostridium sordelli* antitoxin would neutralize *C. difficile* toxin B (4, 16, 17). By the mid 1980s, commercial kits were being developed and tested to allow a cell culture cytotoxicity assay to be used in settings that lacked a comprehensive enteric disease lab (15, 19). Among other cell lines, the Chinese hamster ovary (CHO) cell culture assay for toxin B has come to be viewed as the "gold standard" for clinical diagnosis (endoscopy for detection of mucosal plaques notwithstanding) of *C. difficile* enteritis because of its great sensitivity (50 pg of toxin B per ml of sample) (11), although a standardized protocol is not available (12).

Some question arises, however, in that the CHO assay may have low specificity at low titers of toxin B. A positive cell culture cytotoxin assay with specimens from healthy adults, neutralizable by specific antitoxin, has been reported for 0 to 11.4% of assays (13, 14). Approximately 15% (4 of 26) of rigorously defined "healthy" controls tested concurrently by our laboratory in a separate cohort were positive for the toxin B assay at low titer (1:10). It should be noted that two of these four positive samples were retested with a negative result; however, such retesting is normally not indicated and would not be performed in a clinical lab.

Because we have observed a large number of positive results for the CHO assay at only the 1:10 dilution when testing for *C. difficile* in inpatients, our purpose in this study was to evaluate the frequency at which additional evidence of *C. difficile* diarrhea might be present in these individuals. Specifically, we evaluated the correlation of neutralizable CHO cell cytotoxicity at different titers with a toxin A ELISA, a latex agglutination assay for fecal lactoferrin, and clinical presentation.

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MATERIALS AND METHODS

Stool samples were obtained from two groups of patients for use in this study. The first set were collected during the period December 1989 to February 1991 at the Veterans Affairs Medical Center in Salem, Va. Submissions were unformed stools from inpatients, generally by physician request for *C. difficile* testing, although samples submitted for other tests with sufficient quantity remaining for the *C. difficile* battery were also examined. In all, 359 stools were examined by the CHO cell culture assay for toxin B, an ELISA for toxin A, and a latex agglutination test for fecal lactoferrin.

Additionally, for 123 of the samples, a chart review was performed to evaluate the clinical likelihood of *C. difficile* diarrhea. The following criteria, adapted from Holter et al. (8), were evaluated; if three or more were satisfied, the clinical observations were considered to be consistent with the diagnosis of *C. difficile* diarrhea: (i) antibiotic treatment within 2 months, (ii) significant diarrhea (≥3 stools of changed consistency within a 24-h period), (iii) response to oral vancomycin or metronidazole and/or significant improvement in patient condition after antibiotic discontinuation, and (iv) no other etiology or diarrhea-inducing procedures or medications.

The second set of samples was obtained in a similar manner at the University of Virginia Medical Center in Charlottesville during the period from 9 July to 28 October 1991. In this instance, 363 specimens were examined and 114 charts were reviewed.

All testing protocols and examination criteria were identical for each group of specimens, except for minor variations as noted below. Tests were performed at both study sites. For some patients, more than one sample was collected and examined. Of the 722 total samples, 130 represent multiple specimens obtained from 89 of 681 total patients. Of these 130 samples, 70% (91 of 130) represent samples for which the assay results changed and/or at least 1 week elapsed before evaluation of a subsequent stool sample. All paired analyses were done on the same specimen. The chart reviews were performed for all records which were available at the time of review.

Quantitative CHO cell cytotoxin assay. Stool samples, stored frozen at −70°C (−30°C in the University of Virginia study), were used. Testing was performed as quickly as possible after obtaining the sample, but this freeze-thaw cycle may have caused some loss of cytotoxic activity. Samples were diluted to 1:10 with sterile phosphate-buffered saline (PBS) and centrifuged for 30 min at 1,900 × *g*. Supernatants were filter sterilized by using a 0.22-μm, low-binding filter and were further diluted with PBS to 1:100 and 1:1,000. A neutralization standard was prepared for each sample dilution by adding 25 μl of specific polyclonal *C. difficile* antitoxin (Techlab, Blacksburg, Va.) (6) to 25 μl of sample dilution and incubating for 30 min at room temperature.

To perform the assay, a 96-well, flat-bottom culture plate was prepared with one confluent culture flask of CHO cells in F-12 culture medium containing 1% fetal calf serum and 1% penicillin-streptomycin. Each well was inoculated with 200 μl of the cell suspension and incubated at 37°C in a 5% CO_2 incubator until inoculated. Inoculations included each sample filtrate at 1:10, 1:100, and 1:1,000 dilutions and corresponding neutralization controls (filtrate and antitoxin) at each dilution. The following additional controls were prepared: purified toxin (three dilutions), purified toxin with antitoxin (three dilutions), PBS only, and antitoxin only. The cells were then incubated overnight at 37°C in 5% CO_2. Evaluation of the assay was as follows: negative = <50% cell rounding in stool sample dilution wells; positive = >50% cell rounding in stool sample dilution wells and ≥50% reduction of cell rounding in the corresponding neutralization well.

ToxA test (Techlab) monoclonal ELISA for detection of *C. difficile* toxin A. Frozen fecal samples were thawed, diluted 1:2 with the product diluent (buffered protein solution with 0.02% thimerosal), and mixed for 10 s. Each sample was placed in a flat-bottom assay well coated with toxin A polyclonal antibody. To each well was added the ELISA conjugate, mouse monoclonal antibody for toxin A coupled to horseradish peroxidase in buffered protein solution with 0.02% thimerosal. Positive and negative control wells were also prepared with purified toxin A and diluent without toxin, respectively, prior to addition of the conjugate. The assay wells were covered and incubated for 50 min at 37°C.

After incubation, samples were removed and wells were washed five times with product thimerosal buffer solution. Samples were then incubated for 10 min at room temperature with two substrates, buffered urea peroxide and tetramethylbenzidine. A sulfuric acid intensifier was added after the 10-min incubation, and absolute spectrophotometric A_{450} was measured (negative = ≤0.100; indeterminate = 0.101 to 0.200; positive = >0.200).

Latex agglutination assay for detection of fecal lactoferrin (Leukotest; Techlab) from fecal leukocytes (LFLA). Stool dilutions were prepared at 1:50 and 1:200 for each sample using diluent (buffered protein containing 0.1% sodium azide) and mixed. For each sample in separate wells, the two dilutions were mixed with rabbit anti-human lactoferrin antibody-coated latex beads. The 1:50 dilution was mixed with negative control latex beads and rotated for 3 min. Interpretation was as follows: negative = no or very fine agglutination; positive = definite agglutination.

When samples were tested at the VA hospital, this assay was still being evaluated and kits were not yet available. The materials used, however, were prepared according to Techlab's protocol.

Statistical *P* values were calculated by using chi square analysis with Yates correction from EpiInfo (Centers for Disease Control and Prevention, Atlanta, Ga.).

TABLE 1. Comparison of results from LFLA and ToxA assays with CHO assay results (n = 671)

CHO titer	No. with result of LFLA/ToxA assay[a]				Total
	−/−	+/−	−/+	+/+	
Negative	261	165	11[c]	11[c]	448
1:10	84	55	3	13	155
1:100	1	6	7	25	39
1:1,000	0	1	7	21	29
Total[b]	85	62	17	59	223

[a] −/−, LFLA negative/ToxA negative; +/−, LFLA positive/ToxA negative; −/+, LFLA negative/ToxA positive; +/+, LFLA positive/ToxA positive.
[b] CHO positive titers (1:10, 1:100, 1:1,000) only.
[c] Some CHO cytotoxicity results may have missed low-titer positive specimens (some of which had been frozen), as suggested by the 22 specimens that were ToxA positive, CHO negative, because we tested only at ≥1:10 stool dilutions (instead of 1:4 as done in some laboratories).

RESULTS

As data from both study sites were comparable, the combined data are presented. The results are summarized in Table 1 for the 671 specimens which were tested using both assays and for which results were conclusive (i.e., not indeterminate). Negative CHO assay results were obtained for 448 samples, and positive results were found in the other 223 specimens. Among these 223 positive samples, the breakdown at titers of 1:10, 1:100, and 1:1,000 was 155 (69%), 39 (18%), and 29 (13%).

The ToxA test correlated well with these negative CHO results and was also negative in 95% (426 of 448) of cases. For specimens negative by the CHO assay, 61% (272 of 448) of specimens were also LFLA negative.

Methods and criteria used by other laboratories for CHO cell cytotoxicity testing include requiring 90% rounding with 90% neutralization (instead of the 50% rounding and 50% neutralization we used). On reexamining all 427 CHO assay results from the University of Virginia Medical Center, 140 of 183 that had been CHO positive (106 of 148 at 1:10) remained positive by the 90% rounding and 90% neutralization criteria.

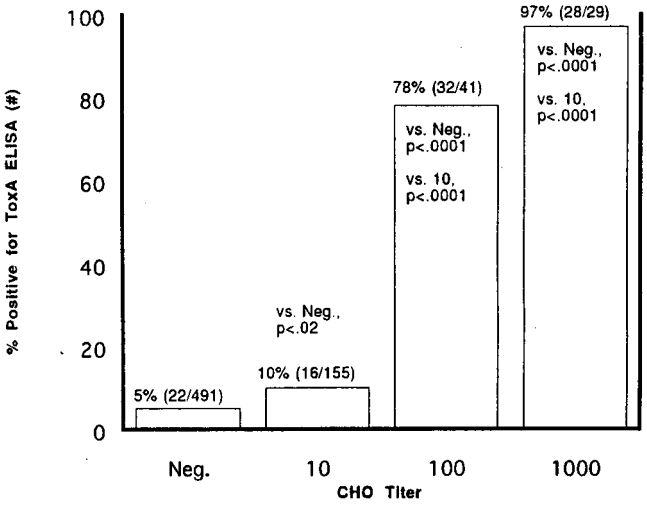

FIG. 1. Comparison of percentages (numbers) positive for toxin A ELISA and CHO cell cytotoxicity titer in patients with suspected *C. difficile* colitis.

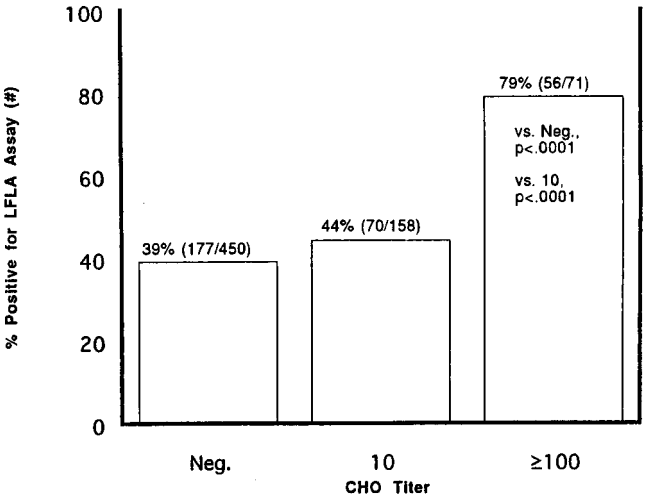

FIG. 2. Comparison of percentages (numbers) positive for lactoferrin latex agglutination assay and CHO cell cytotoxicity titer in patients with suspected *C. difficile* colitis.

While Meridian Premier Toxin A EIA agreed with ToxA results for 32 of 38 (84%) of specimens we tested by all methods, it too was of limited sensitivity, being positive for only 2 of 18 (11%) CHO-positive specimens and negative for 19 of 20 (95% specificity) CHO-negative specimens.

For positive CHO results, it can be seen that as CHO cytotoxicity titer increased, the percentage of specimens with a LFLA-negative/ToxA-negative result decreased, from 54% (84 of 155) at 1:10 to 0% at 1:1,000. Similarly, the percentage of specimens with a LFLA-positive/ToxA-positive result increased, from 8% (13 of 155) at 1:10 to 72% (21 of 29) at 1:1,000.

Shown in Fig. 1 are the significantly increasing percentages of specimens positive by ToxA ELISA with increasing CHO cytotoxicity titer, from 5% (22 of 491) positive by ToxA assay for CHO-negative specimens to 97% (28 of 29) at a CHO titer of 1:1,000 ($P < 0.02$ to $P < 0.0001$). A similar, significant trend is seen with the LFLA assay in Fig. 2, with 39% (177 of 450) of

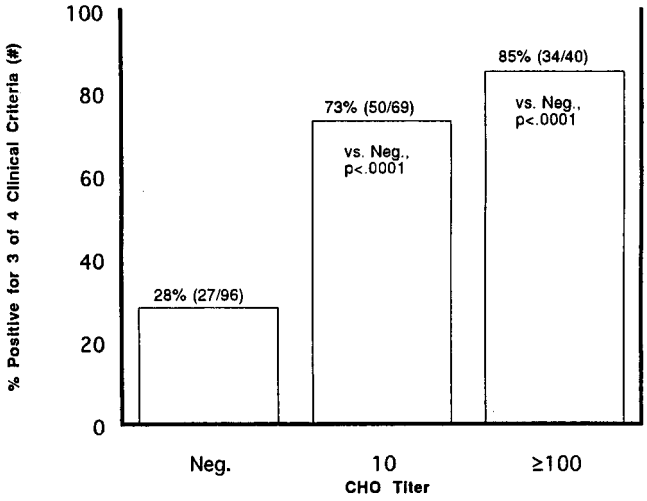

FIG. 3. Comparison of percentages (numbers) positive for three of four clinical criteria and CHO cell cytotoxicity titer in patients with suspected *C. difficile* colitis.

TABLE 2. Specificity and sensitivity values for LFLA and ToxA assays

Assay	Specificity (% [no. tested[a]])	Sensitivity (% [no. tested[a]])
LFLA	61 (273/450)	55 (126/229)
ToxA	96 (467/489)	34 (76/225)

[a] Numbers here include results from specimens not included in Table 1 because of incomplete testing.

specimens positive when the CHO assay was negative and 79% (56 of 71) positive when CHO titer was ≥1:100 ($P < 0.0001$). There was no significant difference between the rates of LFLA-positive samples at a CHO-negative titer and a CHO titer of 1:10. The percentage of individuals meeting three of the four clinical criteria for antibiotic-associated *C. difficile* diarrhea is shown in Fig. 3. Percentages significantly increased from 28% (27 of 96) to 85% (34 of 40) as the CHO cytotoxicity titer increased from negative to ≥1:100 ($P < 0.0001$).

It should be noted that the denominators in Fig. 1 to 3, as well as Fig. 4, differ from each other and Table 1 because of the inclusion of data which were excluded from Table 1 because of partial collection or indeterminate results.

Sensitivity and specificity values for the LFLA and ToxA tests (based on the CHO assay as a standard with a result of ≥1:10 considered positive) can be determined and are shown in Table 2. As expected, the LFLA data had intermediate values for specificity (61%) and sensitivity (55%), indicative of a test that may detect any inflammatory diarrhea. The ToxA test showed high specificity (96%) but had a low sensitivity (34%), indicating many false negatives.

DISCUSSION

The need for rapid, sensitive, and specific detection and diagnosis of *C. difficile* diarrhea is demonstrated by the wide array of new tests developed recently for the detection of toxins and/or the organism (10). *C. difficile* is noted as the cause of antibiotic-associated diarrhea in 15 to 25% of such cases (3). Additionally, asymptomatic carriage of *C. difficile* in healthy adults ranges from 0 to 11.4% (3, 14). In hospitalized patients, asymptomatic carriage has been noted at 10 to 20% (3, 13). Not all antibiotic-associated diarrhea is caused by *C. difficile*, and some persons may harbor *C. difficile* organisms, even toxigenic strains, asymptomatically. Consequently, positive CHO assays which are very sensitive may also give positive results in patients with diarrhea of another etiology, and thus they lack specificity, especially at low titer. The toxin A ELISA in various forms, when compared to a CHO standard, has a specificity of >97% in most studies; its sensitivity typically falls in the range of 65 to 85% (1). However, this low sensitivity may also relate to a lower specificity for the CHO assay, against which the ToxA is being compared in these studies. The lactoferrin assay appears to be sensitive for fecal lactoferrin, characteristic of inflammatory diarrhea, but it is not specific for any single inflammatory enteric pathogen.

The ToxA test was found to have a high (96%) specificity but a low (34%) sensitivity which may limit its usefulness. Additionally, the performance of the ToxA assay is consistent with the published literature (11) indicating cytotoxin titers of 1:100 have toxin A concentrations of 1 ng/ml that should be detectable by ELISAs. There are at least two possible interpretations of these results. First, the ToxA test may indeed lack sensitivity and fail to detect many episodes of diarrhea caused by *C. difficile*. Alternatively, the test to which the ToxA test is compared may lack specificity; the CHO assay may be positive at a

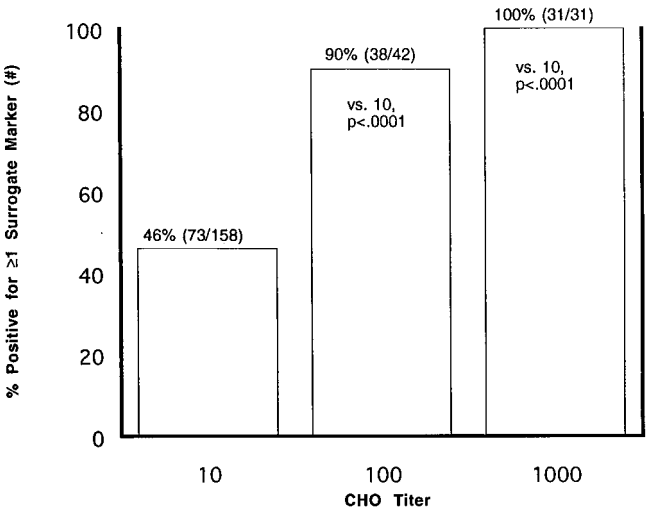

FIG. 4. Comparison of percentages (numbers) positive for ≥1 positive surrogate marker (ToxA, LFLA, clinical criteria) and CHO cell cytotoxicity titer in patients with suspected *C. difficile* colitis.

1:10 stool dilution and not actually detect *C. difficile*-induced diarrhea, thus inflating the number of ToxA false negatives.

The LFLA assay provides a fast and easy test for inflammatory diarrhea. As would be expected, it has a moderate sensitivity and is relatively nonspecific. It remains a useful assay, nonetheless. The sensitivity of LFLA relative to cytotoxin is similar to that published by Yong et al., using enzyme immunoassay for toxins A and B. If data are analyzed for fecal lactoferrin sensitivity relative to ToxA, this value of 71% (70 of 98), is similar to the 75% noted by Yong et al. (20).

The trend toward surrogate markers (ToxA, LFLA, or clinical criteria) being positive at greater frequency with increasing CHO assay titer clearly supports the hypothesis that the CHO assay may be nonspecific. First, the other assays (LFLA, ToxA) and clinical diagnosis support the diagnosis of *C. difficile*-induced diarrhea at high CHO titer (≥1:100) much more often than they do at low CHO titer (1:10). Figure 4 shows that, for combined data, at a CHO titer of 1:10, only 46% (73 of 158) of the samples correlated with another positive surrogate marker (ToxA, LFLA, or clinical criteria). For higher CHO titers, 90% (38 of 42) of samples positive at 1:100 by CHO correlated with a positive surrogate marker. All samples (31 of 31) positive at 1:1,000 by the CHO assay were corroborated by another marker. The difference between the percent corroborated at a CHO titer of 1:10 and a CHO titer of 1:100 or 1:1000 was significant ($P < 0.0001$). This supports the hypothesis that a CHO titer of ≥1:100 significantly and more specifically detects *C. difficile*-associated diarrhea.

Second, if the samples positive by the CHO assay at ≥1:100 are separated for purposes of determining the sensitivity of the ToxA test, the sensitivity of the ToxA assay changes dramatically. For a CHO titer of 1:10, the ToxA sensitivity is 10% (16 of 158), whereas for a CHO titer of ≥1:100, that same value rises to 82% (60 of 73).

Furthermore, the percentage of patients (79%) with a neutralizable CHO cytotoxicity titer of ≥1:100 who have an increased level of fecal lactoferrin (Fig. 2) is remarkably similar to the 75% of *C. difficile*-positive patients with elevated fecal lactoferrin in the Massachusetts General Hospital study (20). Moreover, there was no significant difference between the percent positive rates for the LFLA assay at a negative CHO titer and a positive CHO titer of 1:10. However, for a CHO titer of ≥1:100, the differences compared to specimens which were CHO negative and positive at a titer of 1:10 were significant ($P < 0.0001$).

Finally, as noted earlier, unpublished data showed that in 26 rigorously screened healthy controls participating in a diarrhea study, four (15%) tested CHO positive at titer 1:10, indicating that a portion of normal asymptomatic individuals may carry *C. difficile* or that the CHO assay may lack specificity and detect false positives.

These data indicate that diarrhea associated with a CHO positive assay only at low titer (i.e., 1:10) may not always represent true *C. difficile*-associated diarrhea. A number of etiologies other than or including *C. difficile* diarrhea are possible. (i) Nascent or mild *C. difficile* infection associated with a low titer of toxin and little or no inflammation and (ii) other noninflammatory diarrheas with some overgrowth of *C. difficile* but not to the extent to cause inflammatory diarrhea are two possibilities. Additionally, 39% of patients who are CHO assay negative had positive LFLA tests, which further implicates other inflammatory diarrheas for critical consideration.

In considering present concerns about the emergence of drug-resistant strains of potentially harmful microorganisms, especially vancomycin-resistant enterococci, it has become increasingly important to accurately diagnose *C. difficile*-related diarrheas. We conclude that a positive CHO cell culture assay for *C. difficile* toxin B is associated with corroborating evidence for *C. difficile*-associated diarrhea in a direct relationship as CHO titer increases. While highly sensitive, the CHO assay, at low titer, may lack specificity and should be interpreted in conjunction with additional clinical and laboratory findings.

The toxin A ELISA is highly specific, but, like other toxin A ELISAs, it may lack sensitivity, especially at low titers of toxin. Assays for fecal lactoferrin (polymorphonuclear leukocytes) such as the LFLA test provide additional, independent evidence for inflammatory diarrhea. Clinical findings always merit important consideration in the diagnosis of this disease. A definitive means of diagnosing *C. difficile*-related diarrhea remains elusive, but a careful diagnostic approach which includes a CHO cell cytotoxicity assay, clinical criteria, and other tests can afford greater reliability in diagnosis.

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