# Evaluation of an Enzyme Immunoassay for Detection of the *Clostridium difficile* Common Antigen, Glutamate Dehydrogenase (GDH), in Fecal Specimens

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## Revised Abstract

*C. difficile* is reported to cause up to 25% of cases of antibiotic-associated diarrhea (AAD) and most cases of pseudomembranous colitis (PMC). Laboratory diagnosis typically consists of toxin detection in fecal specimens by enzyme immunoassay (EIA) or tissue culture cytotoxin assay (CTA). Culture on *C. difficile* selective media offers the highest sensitivity, but has low specificity due to detection of non-toxigenic strains and isolates must be tested for toxin production. A comparison of 3 EIA methods was performed on fecal specimens submitted to the UCLA Clinical Microbiology Laboratory from patients with suspected *C. difficile* colitis. EIA kits tested were *C. DIFF-CHEK*™ [(CDAC) (TechLab), EIA for the detection of GDH antigen], Premier™ Toxins A&B (Meridian) and *C. difficile* Toxin A+B Antigen Detection (CTAD) (IVD Research, Inc.). CDAC was evaluated with both the 30 and 60 min protocols. All methods were compared to culture, with resolution of discrepant results using PCR. Of 100 specimens tested, 20 were positive by one or more of the EIA tests and/or culture. Of these, 18 were positive for GDH antigen with both CDAC-30 and CDAC-60, and 17 were positive by culture. No specimen tested positive by culture and negative by CDAC. The Premier™ kit detected toxin in 8 fecal specimens, all of which were positive by both culture and CDAC. Of these 8 specimens, 5 were also positive using CTAD. CTAD was positive for 2 additional specimens, 1 was positive by culture and CDAC, and the other was negative by all methods used. The presence of toxin A&B genes was confirmed in all specimens testing positive by Premier™ and in 6 of the 7 testing positive by CTAD. Seven samples negative by both Premier™ and CTAD were positive for Toxin A&B genes by PCR. PCR detected a Toxin B gene in one sample that was negative for Toxin A&B by both EIA methods. This study demonstrates that CDAC may be a useful screening tool to reduce the number of specimens tested for toxin production. It would be especially useful for laboratories performing CTA, eliminating the need to culture CTA negative specimens and reducing workload related to processing and evaluation of tissue cultures.

## Introduction

- *C. difficile* is a spore-forming, gram-positive anaerobic bacillus first isolated in 1935 from the stool of neonates[1]
  - Etiologic agent in >95% cases of pseudomembranous colitis (PMC) and up to 25% of antibiotic-associated diarrhea (AAD) cases[2]
  - Strains may or may not be toxin-producing and non-toxigenic strains are considered avirulent[2]
- Pathogenesis
  - *C. difficile* is most commonly isolated during or after antibiotic therapy
    - Almost all antibiotics are implicated as a cause of AAD except for aminoglycosides[1]
    - Antibiotics disrupt normal intestinal flora and allows *C. difficile* to become established and proliferate
  - Presence of virulence factors may effect severity of infection and course of disease
    - Production of Toxins A (enterotoxin) and/or B (cytotoxin) cause fluid secretion, inflammation and mucosal damage, leading to AAD or PMC[2]
      - Strains with Toxin A gene deletion and thus produce only Toxin B are uncommon, but can still cause severe disease[1]
    - Role of other virulence factors is speculative
- Clinical Presentation
  - Asymptomatic carriage in small percentage of healthy adult population[1]
    - Asymptomatic carriage rate higher in patients with previous hospitalization or long-term antibiotic use
    - Unknown if carriage rate is associated with transient colonization or is a stable component of normal intestinal flora
  - Symptoms may range from mild diarrhea to PMC with serious sequelae
- Laboratory Testing
  - Typically consists of toxin detection in fecal specimens by EIA or CTA

        - CTA sensitivity <85%, but can be increased to >99% if combine CTA with culture[4]
        - CTA not standardized, requires tissue culture, with 24 hr to 4 day turnaround and interpretation of CPE is subjective
        - When commercial EIA kits are compared to standard CTA [4]
            - EIA sensitivities range from 55.4% to 89.1%
            - EIA specificities range from 89.7% to 99.7%
    - Formed stools should not be tested, except as part of an epidemiologic investigation
    - Other testing methods
        - Culture on selective media: cycloserine cefoxitin fructose agar (CCFA)
        - Antigen detection other than Toxin A or B
        - Metabolic end-product analysis by gas-liquid chromatography (GLC)
        - Molecular methods
- Laboratory Testing
    - Current testing algorithm
        - Initial specimen tested for the presence of *C. difficile* toxin with commercially-available EIA kit
            - If positive, no further testing until therapy complete
            - If negative, one additional specimen accepted within same week
            - If still negative and additional testing requested, then only one specimen accepted per week

## Materials and Methods

- Specimens:
    - Stool specimens used for this study were received for routine laboratory testing for *C. difficile* Toxin A&B
    - Specimens received on weekends, holidays and with insufficient volume were excluded
    - Patient age, sex and specimen consistency (formed/soft/liquid) were recorded upon culture inoculation
        - Pediatric patients (18 years or under) comprised 8.5% of the population studied
- Stool Culture
    - Stool specimens were stored at 2 to 8°C until inoculated onto pre-reduced CCFA (Anaerobe Systems, Morganhill, CA)
        - Culture inoculation and incubation generally occurred within 48 hr of collection
        - Specimens received more than 48 hours after collection were excluded from the study
    - Inoculated media was incubated anaerobically at 35°C for 72 hr
    - Appearance of colony morphology consistent with *C. difficile*
        - Presumptive *C. difficile* colonies on CCFA are large (about 4 mm), yellow, ground-glass in appearance, circular with slight filamentous edge, and are umbonate to flat in profile
        - Growth of *C. difficile* releases characteristic “barnyard” odor
        - Colonies that did not meet all of the above criteria, but were suspicious in appearance, were subcultured to Brucella agar (Remel, Lenexa, KS) and incubated anaerobically as above for further identification
            - Identification as *C. difficile* was confirmed by the presence of chartreuse autofluorescence on Brucella agar and biochemical identification using RapidAna (Remel, Lenexa, KS)
- *C. DIFF-CHEK*™ (TechLab, Inc., Blacksburg, VA): detection of GDH antigen (CDAC)
    - Fecal specimens were stored at 2 to 8°C upon receipt and tested within 72 hr
    - Testing was performed per manufacturer instructions for both CDAC-30 and CDAC-60
- Premier™ Toxins A&B (Meridian): detection of *C. difficile* toxins A and B
    - Fecal specimens were stored at 2 to 8°C upon receipt and tested within 48 hr
    - Testing was performed per manufacturer instructions
- *C. difficile* Toxin A+B Antigen Detection (IVD Research, Inc.): detection of *C. difficile* toxins A and B (CTAD)
    - Aliquots of fecal specimens were stored at -20°C at time of culture inoculation (within 24 hr of collection) and remained frozen until tested
    - Specimen testing was performed per manufacturer instructions
- Polymerase chain reaction
    - Fecal DNA was extracted from 200 μl (or μg) of fecal material using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) and eluted into 200 μL elution buffer
    - DNA amplification was performed with 10μl eluted DNA using Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ).
    - Primers for GDH gene were designed based upon published sequences and amplification parameters were established at TechLab, Inc. (proprietary information)[5]
    - Primers for Toxin A and B genes and amplification parameters were as previously described [6]
    - Amplicons were detected in a 1% agarose gel stained with ethidium bromide
- Phase I: 100 fecal specimens from patients with suspected *C. difficile* colitis were tested for toxin production with the Premier™ Toxin A&B kit, per standard laboratory operating procedures
- Phase II: an additional 211 fecal specimens from patients with suspected *C. difficile* colitis
    - Determine utility of CDAC as a screening tool to triage specimens for additional toxin testing
    - Testing included CDAC, culture, and toxin testing by Premier™ and CTAD (see methods above)

- Discrepant results were resolved by PCR
- Sensitivity and specificity of each assay was evaluated

## Results

- Phase I: 100 fecal specimens tested
  - Only 22 specimens were positive by one or more of the EIA tests, PCR and/or culture

| Phase I | | CDAC-30 | | CDAC-60 | | Premier | | CTAD | | Culture | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Pos | Neg | Pos | Neg | Pos | Neg | Pos | Neg | Pos | Neg |
| Culture | Pos (n=17) | 17 | 0 | 17 | 0 | 8 | 9 | 6 | 11 | N/A | N/A |
| | Neg (n=83) | 2 | 81 | 1 | 82 | 0 | 83 | 1 | 82 | N/A | N/A |
| PCR GDH | Pos (n=20) | 18 | 2 | 17 | 3 | 8 | 12 | 6 | 14 | 16 | 4 |
| | Neg (n=80) | 1 | 79 | 1 | 79 | 0 | 80 | 1 | 79 | 1 | 79 |
| PCR Toxin A | Pos (n=14) | 14 | 0 | 13 | 1 | 7 | 7 | 6 | 8 | 12 | 2 |
| | Neg (n=86) | 4 | 82 | 4 | 82 | 1 | 85 | 1 | 85 | 5 | 81 |
| PCR Toxin B | Pos (n=15) | 15 | 0 | 14 | 1 | 7 | 8 | 6 | 9 | 13 | 2 |
| | Neg (n=85) | 4 | 81 | 4 | 81 | 1 | 84 | 1 | 84 | 4 | 81 |

  - *C. DIFF-CHEK*™ (CDAC)
    - The two GDH PCR positive but CDAC negative specimens were negative by all other methods used in this study
      - The GDH PCR assay is designed to be highly sensitive and will detect DNA from nonviable organisms
      - When compared to culture alone, there were no apparent false negatives
    - The single CDAC positive, GDH PCR negative specimen was positive by culture, and therefore presumed to be a true positive
      - The presence of *Proteus* sp. or other DNAse-producing organisms in stool specimens may be the source of false negative DNA amplification results
    - Using GDH PCR and culture together as the reference, CDAC-60 has sensitivity of 85.7% and CDAC-30 has a sensitivity of 90.5%
      - When compared to culture alone, there were no apparent false negatives
    - Using GDH PCR and culture together as the reference, both assays had a specificity of 98.8%
  - Premier™ Toxins A&B
    - When compared to PCR for toxins A and B, sensitivity is 46.7% and specificity is 98.8%
    - Difficult to assess true sensitivity in the presence of so few positive results
  - *C. difficile* Toxin A+B Antigen Detection
    - When compared to PCR for toxins A and B, sensitivity is 40% and specificity is 98.8%
    - Difficult to assess true sensitivity in the presence of so few positive results
- Phase II: 211 fecal specimens tested
  - 92 of 211 fecal specimens were positive for one or more EIA tests and/or culture
  - Results may not reflect the true incidence since many samples were excluded due to insufficient sample quantity for testing with all methods

| Phase II | | CDAC-30 | | CDAC-60 | | Premier | | CTAD | |
|---|---|---|---|---|---|---|---|---|---|
| | | Pos | Neg | Pos | Neg | Pos | Neg | Pos | Neg |
| Culture &/or GDH PCR | Pos (n=65) | 64 | 1 | 63 | 2 | 46 | 19 | 43 | 22 |
| | Neg (n=146) | 6 | 140 | 13 | 133 | 4 | 142 | 7 | 139 |
| PCR Toxin A/B &/or consensus | Pos (n=58) | 57 | 1 | 56 | 2 | 45 | 13 | 41 | 17 |
| | Neg (n=153) | 13 | 140 | 20 | 133 | 5 | 148 | 9 | 144 |

  - *C. DIFF*-CHECK™ (CDAC)
    - GDH PCR and culture used together as the reference in Phase II
      - CDAC-60 has sensitivity of 96.9 % and CDAC-30 has a sensitivity of 98.5%
      - Both CDAC-30 and CDAC-60 have a specificity of 95.9%
  - Premier™ Toxins A&B
    - When compared to PCR for toxins A and B and/or consensus for all tests performed, sensitivity in Phase II is 77.6% and specificity is 96.7%

- *C. difficile* Toxin A+B Antigen Detection
  - When compared to PCR for toxins A and B and/or consensus for all tests performed, sensitivity in Phase II is 70.7% and specificity is 94.1%
- Phases I and II resulted in 68 specimens that were positive for toxin production by at least one of the Toxin A&B EIA assays
  - Of these, 47 were positive by both toxin assays, 56 were positive by the CDAC-30 assay and 59 were positive by the CDAC-60 assay
- All specimens positive by either of the toxin assays but negative by either of the CDAC assays were also negative by PCR for GDH and Toxin A&B
  - There were an additional 19 specimens positive by PCR for Toxin A and/or B, that were negative by both toxin EIA tests, but positive by CDAC
  - Two samples were PCR positive for Toxin A only, and 3 were positive for Toxin B only
- Whether looking for toxin production by EIA or the presence of toxin DNA by PCR, there were no higher rates of Toxin A or B positivity associated with an age group, sex or specimen consistency

## Conclusions

- EIA methods for toxin detection, although more rapid than CTA, still have relatively low sensitivity
  - Current EIA testing algorithm may be insufficient to detect all cases
- CDAC may be a useful screening tool to reduce the number of specimens tested for toxin production, especially in laboratories performing CTA
  - CDAC-30 has higher sensitivity without reduced specificity, with more rapid turnaround time
  - CDAC positive specimens require additional testing for the presence of toxin A and/or B
    - Use of CDAC eliminates the need to culture CTA negative specimens, and would reduce workload related to processing and evaluation of tissue cultures
- May also be a useful screening tool for follow-up testing with EIA methods
  - Allows for development of alternative testing algorithm
    - One specimen per week tested for the presence of GDH antigen using CDAC-30
    - Specimens positive using CDAC-30 subjected to further testing for toxin production
      - If positive for toxin production, no further testing until completion of therapy
      - If negative for toxin production, then more frequent testing for toxin production allowed
        - Only for patients previously positive by CDAC
  - Alternative algorithm may serve to increase sensitivity of toxin assays
  - Without a controlled study, the impact on workload and laboratory cost is unknown
- A total of 9 specimens (3% of total study population) negative by CDAC-30, were positive for toxin production using either the Premier™ or CTAD assays
  - All of these were PCR negative for GDH, Toxin A or Toxin B DNA
  - Additional testing necessary to determine if these were the result of false positive toxin testing due to cross-reacting substances or false negative CDAC test due to undetectable levels of GDH antigen, with DNA concentrations also below the limit of detection by PCR
- Limitations inherent to this study
  - Comparison was not made to CTA, the standard commonly used for comparative analyses
  - The presence of DNAse-producing bacteria in the stool may result in false negative PCR results
  - Culture results are most reliable when set up within 24 hr of collection
    - Because this was not always feasible, sensitivity and specificity data were calculated against the combined standard of culture in addition to PCR results
  - Further testing not performed to identify substances potentially causing false positive reactions
  - Although any specimen ordered for routine testing for the presence of *C. difficile* toxin was potentially included in the study, specimens with insufficient volume for all tests were excluded
    - Resulting percentage of specimens positive for toxin production was elevated above the expected rates for our facility
    - This would likely effect positive and negative predictive values; therefore, none were reported

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