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Original article

Clinical performance of a multistep algorithm for the diagnosis of *Clostridioides difficile* infection and patient characteristics in a clinical setting

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Abstract

Background: *Clostridioides difficile* infection (CDI) is a significant cause of antibiotic-associated diarrhea and colitis. Its diagnosis relies on clinical presentations confirmed by laboratory investigations.

Objectives: This study aimed to evaluate the performance of current diagnostic tests and examine the characteristics of patients with CDI in a real hospital setting.

Methods: In total, 299 unformed stool specimens were collected and analyzed using the C. DIFF QUIK CHEK COMPLETE and polymerase chain reaction (PCR) assays. Patient data were retrospectively reviewed from medical records.

Results: The C. DIFF QUIK CHEK COMPLETE and PCR assays detected toxigenic *C. difficile* in 16/299 (5.4%) and 37/299 (12.4%) specimens, respectively. The agreement rates between these two assays for detecting *C. difficile* and toxin A/B were 90.3% and 93.0%, respectively. The use of a multistep algorithm with the C. DIFF QUIK CHEK COMPLETE assay, arbitrated by PCR assays, significantly increased the detection of toxigenic *C. difficile* ($P < 0.05$). Among the clinical characteristics of patients, age > 60 years was significantly associated with CDI ($P < 0.05$). However, the duration of antibiotic exposure and antibiotic type were not significantly different between patients with and without toxigenic *C. difficile*. In addition, *C. difficile* diagnostic tests and treatments are inappropriately used among patients presenting with diarrhea of other causes and a history of antibiotic exposure.

Conclusion: A multistep algorithm is a valuable diagnostic tool for CDI, particularly in hospitals without established testing criteria. To prevent the inappropriate utilization of laboratory resources, effective stewardship of *C. difficile* testing is essential.

Keywords: *Clostridioides difficile* infection, glutamate dehydrogenase antigen, toxin A/B.

Clostridioides difficile is an important causative agent of antibiotic-associated diarrhea and colitis in hospitalized patients, particularly in those of advanced age or those who receive long-term antibiotic treatment for non-*C. difficile* infection. Exposure to certain classes of antibiotics, including second- and third-generation cephalosporins, fluoroquinolones, and carbapenems, can increase the risk of *C. difficile* gastroenteritis.[1, 2] Furthermore, the *C. difficile* infection (CDI) rate increases in community populations without history of antibiotic exposure, such as solid-organ recipients of transplantation, patients with inflammatory bowel disease, and patients who had hematopoietic stem cell transplantation.[3] In the healthcare setting, *C. difficile* is transmitted by contacting with the organism or its spores in the environment or by spreading from person to person through the fecal–oral route. The clinical manifestations of CDI vary among hosts and can include asymptomatic, mild, moderate, or severe diarrhea and colitis.[4] The gold standard methods for diagnosing *C. difficile* infection include the cell

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cytotoxicity neutralization assay and toxigenic culture. However, these techniques are labor-intensive and cannot be performed in most microbiology laboratories.[3] Therefore, rapid tests and point-of-care tests that detect toxin A and/or toxin B and the *C. difficile* glutamate dehydrogenase (GDH) antigen have been practically used for CDI diagnosis using enzyme immunoassay (EIA) or immunochromatographic methods.

The GDH antigen is a highly conserved metabolic enzyme and is present at high levels in both toxigenic and nontoxigenic *C. difficile*.[3] Therefore, GDH detection has been widely implemented as a rapid screening test because of its high sensitivity; however, it has poor specificity for toxigenic *C. difficile*.[5] In addition, nucleic acid amplification tests (NAATs) targeting *C. difficile* genes, such as rRNA, triose phosphate isomerase (*tpi*), and toxin A- and B-encoding genes (*tcdA* and *tcdB*), are useful tools for CDI diagnosis.[5, 6] The Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) recommend the use of a multistep algorithm (GDH + toxin EIA, GDH + toxin EIA arbitrated NAAT, or NAAT + toxin EIA) in diagnosing CDI. Specifically, if no consensus has been reached between clinicians and the laboratory at the institutional level regarding stool testing for CDI diagnosis.[3] In addition, in some centers, the implementation of strict consensus criteria for sample acceptance for *C. difficile* testing could not be controlled. The multistep algorithm should be tested in patients with clinical symptoms (≥ 3 unformed stools in 24 hours.) who do not receive laxatives. Moreover, patients who do not meet the aforementioned criteria should not undergo NAAT alone.[3] Clinical evaluation should differentiate CDI from asymptomatic carriage if the results are negative for toxins A and B EIA but positive for GDH or NAAT.[7] The laboratory may use a GDH assay as a rapid screening test, following by or simultaneously with toxin A and B EIA with or without cytotoxin testing, or using NAAT to arbitrate the discrepancy between the GDH and EIA toxin results, which can reduce the turnaround time and costs and improve the diagnostic accuracy.[8]

This study aimed to evaluate the performance of a GDH antigen and toxin A/B combination EIA (C. DIFF QUIK CHEK COMPLETE) arbitrated by NAAT for toxigenic *C. difficile* detection in a real-life setting in a tertiary hospital. The clinical characteristics of patients were also investigated to assess the rationale test request and treatment decisions.

Materials and methods

Clinical specimens and study population

Overall, 299 liquid or unformed stool specimens were prospectively collected from 270 patients with CDI presumed by physicians at King Chulalongkorn Memorial Hospital in Bangkok, Thailand, in 2018–2019. Stool specimens were subjected to routine *C. difficile* toxin A/B detection without requiring additional specimens. The laboratory criteria for specimen recruitment were liquid or unformed stools from patients aged > 2 years. Repeated stool specimens from the same patient within 7 days were excluded. All stool specimens were tested using the C. DIFF QUIK CHEK COMPLETE (TECHLAB, Inc., VA, USA) and in-house polymerase chain reaction (PCR) assays.[6] If the stool specimens were not tested within 24 h after receiving the specimens, they were stored at 4°C and –70°C until testing by C. DIFF QUIK CHEK and PCR, respectively, within 72 h. Patient data regarding characteristics, clinical presentation, onset of unformed stool, frequency of diarrhea per day, history of laxative and antibiotic use in the 8 weeks before the onset of unformed stool, and CDI treatment decisions were analyzed retrospectively from the medical records without any contact with the participants.

This study was approved by the Ethics committee of the institutional review board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA no. 482/2019), which was approved on April 24, 2018. The need for a written informed consent was waived for this study because the study exposed the participants to only minimal risk.

Isolation and identification of C. difficile

An anaerobic culture with alcohol shock enrichment was used to isolate *C. difficile* from 281/299 specimens. Briefly, an equal volume of 95.0% ethanol was mixed with the stool specimens, which were then incubated at room temperature for 45–60 min. The mixtures were plated on brucella blood agar (in-house preparation) and incubated under anaerobic conditions, 5.0% CO_2, and 5.0% H_2 balanced with N_2 (Concept 400, Baker Ruskinn, UK) for 48–72 h.[9] Colonies with typical *C. difficile* characteristics (large, filamentous edges, translucent, and horse barn odor) were chosen and identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (VITEK MS v3.2 system, BioMerieux, France).

Detection of C. difficile and production of toxins A and B

Briefly, 25 µL of stools was tested with C. DIFF QUIK CHEK following the manufacturer's instructions. The sample was diluted, loaded into a well of a cartridge, and incubated for 15 min at room temperature. The wash buffer and substrate were added to remove unbound antigens and develop color, respectively. Results were recorded after 10 min of incubation at room temperature. Positive results for the GDH antigen and toxins A and B were observed as visible bands in specific wells for each target.

Detection of C. difficile tpi, tcdA, and tcdB genes using PCR assays

An in-house PCR assay was utilized to detect *C. difficile* genes: *tpi* housekeeping gene, *tcdA* gene, and *tcdB* gene encoding triose phosphate isomerase, toxin A, and toxin B, respectively. Positive results for *C. difficile* toxin analysis by PCR assays were reported when the *tcdA* and/or *tcdB* genes were detected. *C. difficile* DNA was extracted from the stool specimens. Briefly, a 500 µL of the ASL buffer (stool lysis buffer) (Qiagen, MD, USA) was added to 200 µL of the stool sample, vortex mixed for 3 min, heated at 95°C for 5 min, and centrifuged at 13,000 rpm for 5 min. Moreover, 20 µL of lysozyme (20 mg/mL) was added to the supernatant and incubated at 37°C for 30 min. The mixtures were centrifuged at 13,000 rpm for 1 min, and DNA was then extracted from the 200 µL supernatant with the MagPurix viral/pathogen nucleic acid extraction kit (Zinexts Life Science Corp., Taiwan). In addition, 5 µL of the extracted DNA were amplified by PCR using primers specific to genes encoding toxins A and B (*tcdA*, 369 base pairs; *tcdB,* 160 base pairs) and triose phosphate isomerase (*tpi*, 230 base pairs) for *C. difficile* detection, as described previously.[6] DNA amplification was performed using multiplex PCR master mix (Biotechrabbit GmbH, Germany) with thermal cycles of 95°C for 2 min, 40 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 30 s, and then 72°C for 5 min. PCR products were verified by gel electrophoresis.

Statistical analysis

To investigate the performance of C. DIFF QUIK CHEK, data management and statistical analyses were performed using R software (version 4.2.1). Data were analyzed using descriptive statistics and the chi-square test. Cohen's kappa (agreement: < 0.2, none; 0.2 – 0.4, minimal; 0.4 – 0.6, weak; 0.6 – 0.8, moderate; 0.8 – 0.9, strong; > 0.9, almost perfect) was used to determine the agreement between the assays.[10] Clinical data were analyzed using IBM SPSS version 23.0 (IBM Corp., USA). Means and standard deviations (SD) were used for continuous variables. The comparison of mean differences in outcome variables was performed using a one-way analysis of variance (ANOVA) and the Kruskal–Wallis one-way ANOVA test. Categorical data are shown as frequencies and percentages of formal comparisons using chi-square or Fisher's exact tests, as appropriate.

Results

C. DIFF QUIK CHEK and PCR assays

In total, 299 liquid or unformed stool specimens were collected from 270 patients at King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok, Thailand, from 2018 to 2019. Stool specimens were tested using the C. DIFF QUIK CHEK COMPLETE (C. DIFF QUIK CHEK) assay (n = 299), PCR assay (n = 299), and anaerobic culture (n = 281). Of the 299 specimens, 5.4% (16/299) were positive for both the GDH antigen and toxin A/B (toxigenic *C. difficile*). Most specimens (80.3%, 240/299) were negative for both GDH and toxin A/B (negative for *C. difficile*). In addition, 14.4% (43/299) of the specimens were positive for the GDH antigen alone (nontoxigenic *C. difficile*), and none were positive for toxin A/B but negative for GDH **(Figure 1)**.

The performance of C. DIFF QUIK CHEK in *C. difficile* detection in 281 samples was compared with that of an anaerobic culture as the gold standard. *C. difficile* was isolated and identified in 53/281 (18.9%) stool specimens. C. DIFF QUIK CHEK detected the GDH antigen in 56/281 (19.9%) specimens. The performance rate of C. DIFF QUIK CHEK in *C. difficile* detection was sensitivity, 83.0% (95% confidence interval (CI), 70.2%–91.9%); specificity, 94.7% (91.0%–97.3%); PPV, 78.6% (65.6% – 88.3%); NPV, 96.0% (92.5% – 98.2%); and agreement, 92.5% (88.8% – 95.3%) (kappa = 0.92).

With PCR assay alone, 211/299 (70.6%) specimens tested negative for *tpi*, *tcdA*, and *tcdB* (negative for *C. difficile*). The PCR assay detected

C. difficile tpi, *tcdA,* and *tcdB* in 37/299 (12.4%) specimens [*tcdB* alone in 11/37 (29.7%) and both *tcdA* and *tcdB* in 26/37 (70.3%)], which were reported to be positive for toxigenic *C. difficile*. Furthermore, the PCR assay detected nontoxigenic *C. difficile* in 51/299 (17.1%) specimens that were positive for *tpi* but negative for *tcdA* and *tcdB* genes. The agreement between the PCR assay and C. DIFF QUIK CHEK was 90.3% (kappa = 0.90) for *C. difficile* detection and 93.0% ($K = 0.93$) for toxin A/B detection.

For a hospital without an agreement between the laboratory and physicians regarding the selection of cases to be tested for *C. difficile*, a two-step algorithm should be implemented. This algorithm involves using the C. DIFF QUIK CHEK test as a screening tool, followed by confirmation with PCR assays. Therefore, a two-step algorithm was evaluated. Specimens that tested positive for nontoxigenic *C. difficile* by C. DIFF QUIK CHEK were further examined by PCR assays. The results showed that the two-step algorithm also detected toxigenic *C. difficile* in 15/43 (34.9%) of these specimens **(Figure 1)**. Furthermore, PCR further detected toxigenic *C. difficile* in 6/240 (2.5%) specimens and nontoxigenic *C. difficile* in 23/240 (9.6%) specimens that were negative for *C. difficile* according to the C. DIFF QUIK CHEK test. The two-step C. DIFF QUIK CHEK-PCR algorithm significantly increased the detection of toxigenic *C. difficile* from 16/299 (5.4%) to 37/299 (12.4%) ($P < 0.05$) specimens **(Figure 1)**.

Clinical characteristics and outcomes

A total of 299 stool samples were collected from 270 patients. The median age of the patients was 62 (mean ± SD, 59.8 ± 20.8) years, with ages ranging from 4 to 104 years. Some patients had multiple occurrences of unformed stools at different admissions. Fifteen stool samples were collected during the same episode of those admissions, though > 7 days apart. Therefore, clinical data were analyzed based on 284 stool samples that represented different clinical episodes. Moreover, the final results from a multistep algorithm were categorized into three groups to determine the correlations between *C. difficile* detection results using a multistep algorithm and related clinical data: group A, nontoxigenic *C. difficile* detected (n = 49); group B, toxigenic *C. difficile* detected (n = 36); and group C, *C. difficile* not detected (n = 199). Data assessment results are shown in **Table 1**.

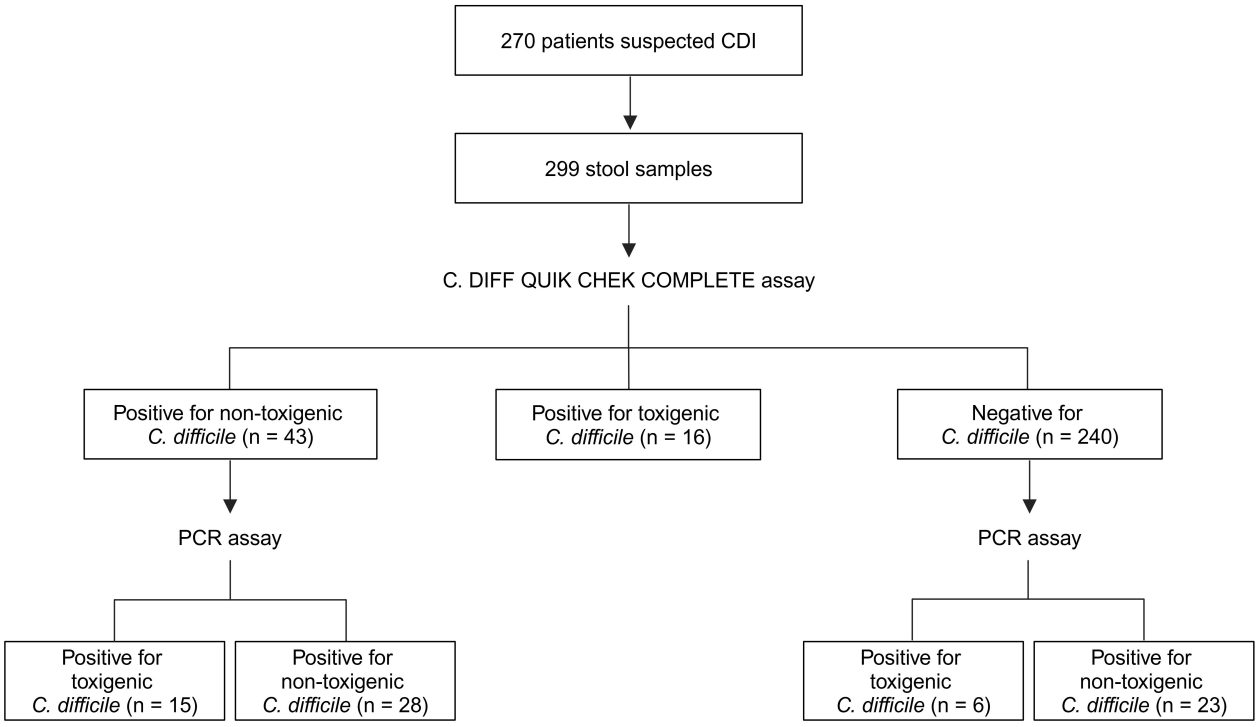

Figure 1. Results of a multistep algorithm using C. DIFF QUIK CHEK and PCR assays in this study. *C. difficile*, *Clostridioides difficile*; n, number; PCR, polymerase chain reaction.

Table 1. Characteristics of the patients based on multistep algorithm of *C. difficile* detection.

Characteristics	Group A Non-toxigenic *C. difficile* detected (n = 49)	Group B Toxigenic *C. difficile* detected (n = 36)	Group C *C. difficile* not detected (n = 199)	*P* - value
Age (years), Mean (SD)	58.6 (23.4)	71.6 (15.7)	57.5 (20.4)	< 0.01
Sex, Male, n (%)	21 (42.9)	20 (55.6)	94 (47.2)	0.505
Patient settings, n (%)				
IPD	45 (91.8)	35 (97.2)	188 (94.5)	0.549
OPD	4 (8.2)	1 (2.8)	11 (5.5)	
Onset of unformed stool after admission for IPD cases (days), mean (SD)	29 (57.1)	17.4 (18.5)	21.4 (35.0)	0.354
Met criteria defining diarrhea; ≥ 3 times of unformed stool/day without any laxatives, n (%)	24 (49.0%)	24 (66.7)	108 (54.3)	< 0.01
Antibiotic exposure in previous 8 weeks, n (%)	38 (77.6%)	32 (88.9)	159 (79.9)	0.379
Accumulative length of antibiotic exposure in previous 8 weeks, mean (SD)	16.6 (12.0)	13.3 (9.8)	17 (30.1)	0.755
Subgroup of antibiotic class exposure, n (%)				
Beta-lactams	33 (67.3)	23 (63.9)	129 (64.8)	0.933
Carbapenems	16 (32.7)	14 (38.9)	67 (33.7)	0.807
Fluoroquinolones	3 (6.1)	5 (13.9)	21 (10.6)	0.484
Clindamycin	3 (6.1)	1 (2.8)	5 (2.5)	0.227
Vancomycin (IV)	2 (4.1)	5 (13.9)	15 (7.5)	0.692
Other(s)	8 (6.2)	4 (11.1)	24 (12.1)	0.697
CDI treatment received, n (%)	7 (14.3)	23 (63.9)	29 (14.6)	< 0.01

n, number; SD, standard deviation; IPD, inpatient department; OPD, outpatient department

By the mean age, group B (71.6 years) was significantly older than groups A (58.6 years) and C (57.5 years) ($P < 0.01$). In addition, the proportions of patients aged > 60 years were 77.8%, 51.0%, and 47.7% for groups B, A, and C, respectively ($P < 0.01$). Stool samples were primarily collected from patients in the in-patient department after 2 weeks of admission. The onset of unformed stool after admission was not different among the three groups. Based on the standardized definition of diarrhea as the new onset of unformed stool occurring ≥ 3 times or >250 g per day without the use of any laxatives[11], only 49.0%, 66.7%, and 54.3% of patients in groups A, B, and C, respectively, met these criteria before the request for *C. difficile* testing. Most patients in all groups had a history of antibiotic exposure in the previous 8 weeks, with average exposure durations of 16.6, 13.3, and 17 days for groups A, B, and C, respectively. Beta-lactams and carbapenems were the two most common antibiotics in the study. However, no significant difference in any type of antibiotic exposure was found among these three groups. For CDI treatment, most patients (63.9%) with toxigenic *C. difficile* (group B) were prescribed antibiotics. However, 14.3% of the patients detected with nontoxigenic *C. difficile* (group A) and 14.6% without evidence of *C. difficile* detection (group C) also received CDI treatment without stewardship policy. Meanwhile, 36.1% of patients with toxigenic *C. difficile* detection (group B) did not contract CDI because of the presence of other clinical conditions that physicians judged to be a more probable cause of unformed stools or diarrhea, such as bacterial sepsis, salmonellosis, and gastrointestinal vasculitis.

Discussion

Currently, CDI is diagnosed based on multiple criteria, including clinical presentation and microbiological laboratory results. The preferred clinical criteria for the suspicion of CDI or *C. difficile*-associated diarrhea is a new onset ≥ 3 diarrheal bowel movements in the 24 h before stool collection, or diarrhea with abdominal pain or cramping.[3] To avoid overdiagnosis or misdiagnosis, the IDSA and SHEA recommend using a multistep algorithm for *C. difficile* diagnosis. This algorithm can detecting GDH plus toxin A/B and GDH plus toxin A/B with arbitration by NAAT, or NAAT plus toxin A/B. In this study, only liquid or unformed stools from patients aged > 2 years were accepted for testing according to the IDSA/SHEA guidelines because infants and young children can have asymptomatic colonization with toxigenic or nontoxigenic *C. difficile* strains.[3] Therefore, unless other infectious and noninfectious causes of diarrhea have been ruled out, these *C. difficile* tests should not be performed.

In this study, the C. DIFF QUIK CHEK showed low sensitivity compared with the PCR assay. Therefore, C. DIFF QUIK CHEK should be arbitrated by NAAT targeting toxin genes if the results for GDH and toxin detection are discordant or negative in specimens of patients with highly suspected CDI. The advent of the GDH assay has enhanced the detection of toxigenic *C. difficile* through a combination with direct toxin detection or NAAT.[12] CDI screening by the detection of GDH antigens in stool specimens had high sensitivity and specificity comparable to that of the standard culture method and NAAT for *C. difficile* detection,[13 - 15] which was consistent with our findings of the high sensitivity and specificity of GDH detection by C. DIFF QUIK CHEK compared with those of anaerobic culture. In addition, an additional possible explanation for the low rate of positive toxin detection in our hospital compared with that using the NAAT method could be the low levels of toxins in the stool specimens and their instability because of inappropriate temperature control, which is sometimes encountered in real clinical settings. The recommendation is to transport stool specimens to a laboratory as soon as possible after collection, and specimens should be stored at 4°C.[16] Moreover, this could be explained by the various strains of toxigenic *C. difficile*, which can affect *C. difficile* detection by EIA assays. [17]

Negative results for both nontoxigenic and toxigenic *C. difficile* strains by a rapid screening test cannot definitively rule out toxigenic *C. difficile* infection, which can lead to incorrect decisions for CDI management. Therefore, NAATs may be necessary for patients with a history and clinical presentation of CDI to confirm and decrease false-negative results of rapid screening tests. The present findings showed that PCR assays could further detect toxigenic *C. difficile* in some of these negative specimens owing to its high sensitivity, as reported previously.[18] However, NAATs can lead to the overdiagnosis of *C. difficile* infection if used alone, particularly in patients without diarrhea from CDI, as they may be colonized with of *C. difficile* without causing infections.[3] A previous systematic review concluded that test performance is related to pretest prevalence and stool sample selection. [19] However, recent cohorts have demonstrated that improvements in *C. difficile* test stewardship have also affected the CDI rate. Moreover, the implementation of a computerized clinical decision support (CCDS) tool significantly reduced *C. difficile* testing. [20, 21] To our knowledge, this study was the first to report test request data in real clinical practice in Thailand, revealing that only half of the patients whose stool specimens were requested for *C. difficile* testing exhibited absolute diarrhea. Some patients solely had a history of antibiotic exposure with episodic unformed stools, and the frequency did not meet the criteria defining diarrhea. This finding suggests an overutilization of the *C. difficile* diagnostic test in real clinical settings, which was observed in other clinical settings, [22, 23] and can affect the *C. difficile* diagnostic test performance. Importantly, physicians might decide on CDI treatment based solely on the presence of unformed stools without other explainable causes. However, extensive investigations were often unavailable. On the contrary, they might not prescribe CDI treatment among patients who had other obvious causes of diarrhea, despite toxigenic *C. difficile* detection. Therefore, the use of a multistep algorithm involving GDH and/or toxin detection plus NAAT is recommended, and results should always be considered alongside the patient's clinical history, presentation, and other laboratory investigations. Moreover, physicians should rationally request *C. difficile* testing, or the CCDS tool should be implemented in Thailand to improve the clinical benefits and decrease laboratory costs and workloads.

Conclusion

A multistep or two-step algorithm using GDH and/or toxin detection and NAAT is recommended in clinical settings, particularly in hospitals without established criteria for testing. This approach is useful, affordable, and convenient. However, physicians should avoid inappropriate testing in cases that do not meet the diarrhea-defining criteria and always interpret test results alongside clinical data to reduce overtreatment in cases with diarrhea from other causes.

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Conflict of interest statement

All authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

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