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Comparison of Three Clostridioides difficile Infection Detection Methods: Postprint

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Abstract

Objective: To evaluate the diagnostic efficacy of three detection methods for Clostridium difficile infection (CDI) and to identify an appropriate diagnostic protocol for CDI. Methods: Seventy stool specimens from patients with diarrhea suspected of CDI were collected at Guangzhou General Hospital of Chinese People' s Liberation Army from May to December 2016, and detected using three methods: (1) culture method; (2) enzyme-linked fluorescence assay for Clostridium difficile toxins A/B (CDAB) and glutamate dehydrogenase (GDH); (3) q-PCR amplification of Clostridium difficile-specific gene tpi and toxin genes (tcdA/tcdB). Using the culture method results as the reference standard, the diagnostic indices of the three methods were calculated. Results: Of the 70 stool samples collected, Clostridium difficile was isolated in 13 cases (18.57%), including 6 toxigenic strains (8.57%). The q-PCR method identified 17 cases positive for the tpi gene, with sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic concordance rate of 92.31%, 91.23%, 70.59%, 98.11%, and 91.43%, respectively, all higher than those of the GDH method (84.62%, 84.21%, 55.00%, 96.00%, 84.29%) (2=24.881, P<0.001). Moreover, the sensitivity of q-PCR amplification of tcdA/tcdB (66.67%) was superior to that of CDAB (33.33%) (2=35.918, P<0.001). Conclusion: CDAB detection and q-PCR showed high specificity, the GDH method had good sensitivity, and all three demonstrated high negative predictive values. Compared with other detection methods, q-PCR for CDI detection offers advantages such as rapid turnaround time, high sensitivity, and good specificity, making it suitable for clinical implementation.

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Full Text

Preamble

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Abstract

Objective: To evaluate the diagnostic efficacy of three detection methods for *Clostridium difficile* infection (CDI) and identify an optimal diagnostic protocol. **Methods:** Seventy stool specimens were collected from patients with suspected CDI at the General Hospital of Guangzhou Military Command from May to December 2016. Three detection methods were employed: (1) culture method; (2) enzyme-linked fluorescent assay for *C. difficile* toxins A/B (CDAB) and glutamate dehydrogenase (GDH); and (3) q-PCR amplification of *C. difficile*-specific gene *tpi* and toxin genes (*tcdA/tcdB*). Using culture results as the reference standard, diagnostic parameters were calculated for each method. **Results:** Among 70 stool samples, *C. difficile* was isolated in 13 cases (18.57%), including 6 toxigenic strains (8.57%). q-PCR identified 17 *tpi*-positive specimens, with sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of 92.31%, 91.23%, 70.59%, 98.11%, and 91.43%, respectively—all significantly higher than GDH (84.62%, 84.21%, 55.00%, 96.00%, 84.29%; ^{2}=24.881, P<0.001). q-PCR amplification of *tcdA/tcdB* also demonstrated superior sensitivity (66.67%) compared to CDAB (33.33%; ^{2}=35.918, P<0.001). **Conclusion:** CDAB detection and q-PCR exhibit high specificity, while GDH offers good sensitivity, and all three methods demonstrate high negative predictive values. Compared to other methods, q-PCR offers advantages in timeliness, sensitivity, and specificity, making it suitable for clinical implementation.

Keywords: *Clostridium difficile* infection; laboratory techniques and methods; diagnostic criteria

Introduction

Clostridium difficile (CD) is a Gram-positive, obligate anaerobic, spore-forming bacillus widely present in nature. Under normal conditions, CD is non-pathogenic; however, extensive antibiotic use or immunosuppression can disrupt the intestinal microbiota, enabling CD colonization or fecal-oral transmission to cause clinical manifestations ranging from mild diarrhea to severe toxic megacolon, colonic perforation, and even death. Accurate and rapid diagnosis of CDI has become a critical challenge for clinical prevention

and treatment. The 2010 clinical practice guidelines from the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America recommend laboratory diagnostic criteria for CDI as detection of toxigenic CD or its toxins in stool, or histopathological confirmation of pseudomembranous colitis.

Current laboratory methods for CDI diagnosis include culture, enzyme immunoassay, and molecular biology techniques. In China, only a few hospitals perform CDI testing, primarily using enzyme immunoassay for toxins A/B (CDAB). However, CDAB suffers from low sensitivity, failing to meet clinical demands for accurate diagnosis. As early as 2001, domestic literature reported using conventional PCR to detect CD with positivity rates up to 46.7%, while culture positivity was only 19.2% (7/36). With technological advances, q-PCR has been applied clinically for *Mycobacterium tuberculosis* detection due to its high sensitivity and specificity, yet its use for CDI remains limited. Therefore, this study aimed to evaluate the diagnostic performance of three CDI detection methods—CDAB testing, GDH testing, and q-PCR for *C. difficile*-specific gene *tpi* and toxin genes (*tcdA/tcdB*)—using toxigenic strain culture as the diagnostic gold standard, and to propose a rapid, sensitive, specific, and cost-effective detection protocol.

Methods

1.1 Specimen Source

Seventy stool specimens were collected from hospitalized patients with 3 watery stools in a single day and clinically suspected CDI at the General Hospital of Guangzhou Military Command between May and December 2016. Enzyme-linked fluorescent analysis was used to detect CDAB and GDH, while q-PCR was employed to detect the specific gene *tpi* and toxin-related genes. Toxigenic strain culture served as the gold standard for CDI diagnosis.

1.2 Reagents and Instruments

The study utilized VIDAS CDAB/GDH reagent kits and the VIDAS fluorescence immunoanalyzer series, ChromID chromogenic culture medium for *C. difficile*, MALDI-TOF MS Vitek-MS (bioMérieux), stool genomic DNA extraction kits (Tiangen Biotech), an anaerobic culture system (Yoder), PCR amplifier (BIO-RAD), Columbia blood agar plates (Huankai Microbiology), *C. difficile* ATCC-BAA-2155 (ATCC), and PCR reagents (Takara).

1.3 Fecal Culture for *C. difficile*

Appropriate stool specimens were mixed 1:1 with anhydrous ethanol, incubated for 1 hour, then inoculated onto Columbia blood plates and *C. difficile* chromogenic plates. Cultures were placed in an anaerobic jar filled with mixed gas to achieve 0.1% oxygen content and incubated at 35°C for 48 hours. Sus-

pected colonies were isolated and purified based on morphology on blood and chromogenic plates, followed by oxygen tolerance screening.

1.4 Identification of Culture-Positive Strains

Initial screening was performed based on Gram stain morphology and characteristic stable odor. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for species identification of suspicious colonies. Bacterial DNA was extracted using the heat shock method to amplify *C. difficile*-specific fragment *tpi* and toxin genes (*tcdA/tcdB*) for confirmation. The PCR amplification system consisted of 1 L each of forward and reverse primers (10 mol/L), 1 L template, 10 L 5× PCR mix, and 7 L deionized water. Reaction conditions were: 94°C for 30 s, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with final extension at 72°C for 5 min. Primers are listed in .

1.5 Enzyme Immunoassay for CDAB and GDH

Fresh stool specimens (200 mg) were mixed with 1 mL of CDAB or GDH specimen diluent, vortexed, centrifuged, and 300 L of supernatant was applied to respective reaction strips for measurement. For CDAB, values <0.13 were considered negative and 0.13–0.37 positive. For GDH, values <0.1 were negative and >0.1 positive. Both assays were calibrated and quality controlled according to kit instructions.

1.6 q-PCR Detection of *C. difficile* and Toxin Genes

Stool specimens (180–220 mg) were processed using the stool genomic DNA extraction kit to obtain genomic DNA (gDNA) as template. TaqMan probe-based qualitative triplex qPCR was performed to detect *tpi*, *tcdA*, and *tcdB*. The 10 L reaction system contained: 2× Master Mix 5.0 L, forward and reverse primers (10 mol) 0.3 L each, template (1 ng/ L) 1.0 L, and deionized water 3.4 L. Reaction conditions were: 95°C pre-denaturation for 5 min, 40 cycles of 95°C denaturation for 15 s and 60°C annealing/extension for 30 s, followed by 95°C for 15 s and 60°C for 1 min. Result interpretation criteria were: (1) *tpi* and *tcdB* Ct values <40 indicated toxigenic *C. difficile* positivity; (2) *tpi* Ct <38 with no *tcdB* amplification indicated non-toxigenic *C. difficile* positivity; (3) *tpi* Ct 38–40 with no *tcdB* amplification required retesting at higher concentration; (4) no amplification of *tpi* or *tcdB* with normal internal control amplification indicated *C. difficile* negativity; (5) no amplification of *tpi* or *tcdB* with internal control Ct >36 or no Ct indicated invalid results. Primers are listed in .

Statistical Analysis

Data were analyzed using SPSS 20.0. Paired chi-square tests compared positivity rates among the three detection methods, with P<0.05 considered statisti-

cally significant. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated.

Results

2.1 Comparison of Three Detection Methods with Reference Standard

A total of 70 stool specimens from suspected CDI patients were collected. Culture isolated 13 *C. difficile* strains, including 6 toxin-producing strains, with 57 negative specimens (). CDAB testing yielded 6 positive, 3 gray-zone, and 61 negative results (). GDH was positive in 20 and negative in 50 specimens. q-PCR detected *tpi* gene positivity in 17 specimens, with 7 showing dual toxin gene *tcdA/tcdB* positivity ().

2.2 Combined Detection Results Compared with Reference Standard

Using toxigenic strain culture as the gold standard, q-PCR(*tpi*) combined with CDAB and GDH combined with CDAB were compared, with positivity defined as detection by at least one method ().

2.3 Diagnostic Indicators of Individual and Combined Methods

Diagnostic parameters including sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy were calculated from data in Tables 3-5. The optimal diagnostic indicators with statistical significance are bolded and underlined (,).

2.4.1 Fecal Culture Characteristics

On Columbia blood plates, *C. difficile* appeared as gray-white colonies with irregular edges that became grayer with deeper staining at the margins upon prolonged air exposure ([Figure 1: see original paper]A). On chromogenic plates, *C. difficile* formed black, shiny colonies with irregular edges ([Figure 1: see original paper]B).

2.4.2 Toxigenic Strain Identification

The *C. difficile*-specific gene *tpi* assists mass spectrometry confirmation of isolated strains, while *tcdA/B* encode toxin genes. DNA was extracted from 13 MALDI-TOF MS-identified *C. difficile* isolates for PCR amplification of *tpi* and *tcdA/tcdB* ([Figure 2: see original paper]). All 13 strains contained the *tpi* gene, confirming their identity as *C. difficile*. Among the 6 toxigenic strains, 5 were A B type and 1 was A B type, with the remainder being non-toxigenic.

Discussion

Clostridium difficile infection (CDI) is recognized as a leading cause of antibiotic-associated diarrhea. Despite implementation of various infection prevention and

transmission reduction measures by health authorities, CDI rates remain high. Accurate CDI diagnosis continues to challenge clinical management.

This study employed both Columbia blood agar and *C. difficile* chromogenic plates for isolation, with MALDI-TOF MS for definitive identification as the reference standard. Among 70 stool specimens, we identified 13 *C. difficile* isolates (6 toxigenic, 7 non-toxigenic), achieving an isolation rate of 18.57%. This represents a significant improvement over previous domestic studies using conventional blood plates alone: Shanghai Huashan Hospital (2008) reported 9.4% isolation from 587 specimens; Cheng et al. in Beijing (2009) found 10.7% positivity in 112 specimens; Guangzhou Medical College (2013) isolated *C. difficile* in 6.2% of 467 cases; and our group previously reported 8.44% isolation from 675 adult diarrhea specimens (2014–2015). The enhanced isolation rate in the current study likely reflects the combined use of chromogenic plates and MALDI-TOF MS.

The most common CDI detection method is enzyme-linked fluorescent analysis for bacterial toxin CDAB. In this study, CDAB positivity was 8.57% (6/70), consistent with domestic reports (8.70%, 8/92) but significantly lower than international data (15.86%, 88/555). Three gray-zone specimens required clinical correlation. Notably, one CDAB-positive specimen cultured *Clostridium perfringens* rather than *C. difficile*. Literature reports indicate that *C. perfringens* toxin TpeL shares 30–39% amino acid homology with *C. difficile* toxins A (TcdA) and B (TcdB), and the two species may act synergistically in intestinal disease. Repeated culture of this specimen yielded only *C. perfringens*, which produced a CDAB value of 0.27 (gray-zone) after 48-hour incubation. Thus, *C. perfringens* may cause false-positive CDAB results. Additionally, improper specimen storage and transport can lead to protein degradation or antigenic variation, potentially affecting detection rates.

Glutamate dehydrogenase is a conserved antigen abundantly present on the *C. difficile* surface, offering high stability and sensitivity with a key advantage of excellent negative predictive value. Meta-analyses report GDH negative predictive values of 94.6–100%. In this study, GDH demonstrated 84.62% sensitivity, 84.21% specificity, and 96.00% negative predictive value, consistent with existing literature.

This study employed q-PCR to simultaneously amplify *tpi*, *tcdA*, and *tcdB*. We detected 17 *tpi*-positive and 7 toxin gene-positive specimens, yielding a 10% toxigenic *C. difficile* positivity rate. Concordance with the reference standard was 91.43% for *tpi* and 92.86% for toxin genes. The negative predictive value for *tpi* detection (98.11%) exceeded that of GDH (96.00%), and toxin gene amplification showed significantly superior diagnostic value compared to CDAB. A 2014 study from Ruijin Hospital using the Xpert *C. difficile* assay for *tcdB* reported 87.2% sensitivity, 92.2% specificity, 81.0% positive predictive value, and 94.9% negative predictive value compared to culture—similar specificity to our q-PCR method. Our simultaneous amplification of both *tcdA* and *tcdB* enables detection of clinically rare but important A B strains. The *tpi* gene

also facilitates screening for non-toxigenic *C. difficile* carriers for subsequent research applications. Etienne-Mesmin et al. demonstrated correlation between fecal toxin gene levels detected by q-PCR and cytotoxicity assays in murine colonization studies. Combined detection of *tpi* and toxin genes by q-PCR meets clinical needs for rapid, accurate diagnosis. However, our q-PCR method still produced some false positives, warranting validation with larger sample sizes and optimization of primer sequences for enhanced specificity.

Retrospective chart review revealed that all culture-positive patients had received long-term proton pump inhibitors combined with imipenem and tigecycline. Extensive research confirms that CDI development correlates with multiple risk factors including advanced age (>65 years), prolonged hospitalization, inflammatory bowel disease, immunosuppressant use, proton pump inhibitors, and broad-spectrum antibiotics. Enhanced CDI surveillance is warranted for patients with these risk factors. For suspected CDI cases, q-PCR screening can provide rapid diagnosis, with chromogenic plate culture used for definitive confirmation.

In conclusion, compared to other detection methods, q-PCR of stool specimens offers superior timeliness, sensitivity, and specificity for *C. difficile* detection, making it suitable for widespread clinical implementation.

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