Clinical evaluation of a non-purified direct molecular assay for the detection of *Clostridioides difficile* toxin genes in stool specimens

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Abstract

Recently, a new rapid assay for the detection of tcdB gene of *Clostridioides difficile* was developed using the GENECUBE. The assay can directly detect the tcdB gene from stool samples without a purification in approximately 35 minutes with a few minutes of preparation process. We performed a prospective comparative study of the performance of the assay at eight institutions in Japan. Fresh residual stool samples (Bristol stool scale ≥5) were used and comparisons were performed with the BD MAX Cdiff assay and toxigenic cultures. For the evaluation of 383 stool samples compared with the BD MAX Cdiff assay, the sensitivity, and specificity of the two assays was 99.0% (379/383), 98.1% (52/53), and 99.1% (327/330), respectively. In the comparison with toxigenic culture, the total, sensitivity, and specificity were 96.6% (370/383), 98.0% (51/60), and 98.8% (319/323), respectively. The current investigation indicated the GENECUBE *Clostridioides difficile* assay has equivalent performance with the BD MAX Cdiff assay for the detection of tcdB gene of *C. difficile*.

Introduction

*Clostridioides difficile* is a Gram-positive, rod-shaped, obligate anaerobic bacterium.
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Competing interests: This study was financially supported by TOYOBO Co., Ltd. The GENECUBE assay and the fee for the BD MAX assay were provided by TOYOBO Co., Ltd. Yosuke Kawashima, and Akio Sugiyama are employees of TOYOBO Co., Ltd. Shigeyuki Nokate received a lecture fee from TOYOBO Co., Ltd. Hiromichi Suzuki received a lecture fee and advisory fee from TOYOBO Co., Ltd. This study was concurrently performed with the clinical testing and the study was performed on the anonymized residual stool samples.

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This study was performed to evaluate the clinical performance of the GENECUBE assay for the detection of toxigenic C. difficile. Several molecular assays for detection of toxin genes have been developed and are classified into three groups: those that detect tcdB gene for the diagnosis of CDI, including the BD MAX Cdiff assay [7]; those that detect cdt gene and tcdC mutations in addition to tcdB gene, including the Xpert C. difficile [9] assay and the Verigene CDF Panel [10]; and multiplex molecular assays such as the FilmArray GI panel [11] and the xTAG Gastrointestinal Pathogen Panel [12]. Most molecular assays have been reported to have excellent performance for the detection of tcdB [7, 13] and prompt identification was reported among several assays [14, 15].

GENECUBE (TOYOBO Co., Ltd., Osaka, Japan) is a Qprobe-PCR based fully automated rapid genetic analyzer capable of extracting nucleic acids from biological material, preparing reaction mixtures, and amplifying a target gene by PCR. This device can handle a maximum of eight samples at once and analyze up to four items at the same time. In the GENECUBE system, purification mode, amplification mode or both modes can be selected for each assay; amplification mode is used for PCR of purified samples or direct PCR of prepared samples. GENECUBE is used for Mycobacterium tuberculosis [16], Mycobacterium avium, Mycobacterium intracellulare, Neisseria gonorrhoeae [17], Chlamydia trachomatis, and Mycoplasma pneumoniae [18, 19]. In addition, assays for the determination of Staphylococcus aureus and mecA were released [20] and rapid precise molecular identification of the causative pathogens from positive blood culture medium without a purification process was reported.

Recently, a new assay for the detection of tcdB of C. difficile with the GENECUBE was created by TOYOBO Co., Ltd. The assay can be performed in approximately 35 minutes with a few minutes of preparation process without a purification. In this study, we performed a multicenter study to evaluate the new C. difficile assay.

Materials and methods
Study design (samples and strains)
This study was performed to evaluate the clinical performance of the GENECUBE Clostridoides difficile assay for the detection of tcdB in stool samples. Comparisons were performed with the BD MAX Cdiff assay (Becton Dickinson and Company, Ltd., New Jersey, USA) and toxigenic cultures (Fig 1).

Fresh residual stool samples (Bristol stool scale ≥ 5 [21]), which were submitted for the evaluation of CDI, were obtained from eight hospitals (Hiroshima University Hospital; HUD, St. Marianna University School of Medicine Hospital; SMD, University of Tsukuba Hospital; TUD, University of Fukui Hospital; FUD, Chutoen General Medical Center; CTD, Tsuruga Municipal Hospital; TRD, Tone Chuo Hospital; TCD, and Tsukuba Medical Center Hospital; TMD) between November 2018 and March 2019. All the stool samples were anonymized after clinical testing and the study was performed on the anonymized residual stool samples.

In the first evaluation, the GENECUBE assay evaluation and C. DIFF QUIK CHEK COMPLETE (QUIK CHEK, Abbott Diagnostics Medical Co., Ltd., Illinois, USA) assay evaluations were performed at each institution. If each institution routinely used the QUIK CHEK for the evaluation of CDI on a daily basis, we used these results in the current study. After the first
evaluation, registered stool samples were transported in cool conditions (2–8˚C) for the second evaluation to SRL Inc. (Tokyo, Japan) for BD MAX assay evaluation with the BD MAX Cdiff assay and to Miroku Medical Laboratory Inc. (Nagano, Japan) for toxigenic culture. SRL Inc. was the only commercially available centralized laboratory in Japan to accept stool samples for the molecular assay evaluation of toxin genes of *C. difficile* in 2018. BD MAX assay evaluation with the BD MAX Cdiff assay was performed as per the manufacturer’s instruction. Positive control and negative control were examined for each evaluation.

The GENECUBE assay evaluation with GENECUBE *C. difficile* assay was performed within 3 days after the submission of stool samples from wards. BD MAX assay evaluation with the BD MAX Cdiff assay and toxigenic culture were performed within 5 days after the submission of stool samples. Assay evaluation of stool samples with insufficient stool volume or with an excess of due date were excluded.

This study was approved by institutional review boards of Hiroshima University Hospital (protocol no. E1395-1) and of each hospital. All assay evaluations were performed after approval.

**GENECUBE assay evaluation with the GENECUBE *C. difficile* assay**

For sample preparation, approximately 20–50 μL stool sample was obtained with a single-use cotton swab and samples were diluted with 1 mL of lysis buffer in filter-equipped tubes (Fig 2).
After filtration of the diluted stool samples, 200 μL diluted stool samples were treated by bead-beating for 20 s with easy beads (TOYOBO Co., Ltd.) for DNA extraction and then centrifuged for 3 mins at 13,000 ×g after the addition of 200 μL of lysis buffer. Then 20 μL supernatant was used for the assay evaluation.

The PCR conditions were as follows: denaturation at 97˚C for 15 s, and 60 cycles of 97˚C for 1 s, 54˚C for 5 s and 63˚C for 2 s. The PCR products were subjected to a melting point analysis, the conditions of which were: 94˚C for 30 s and 39˚C for 30 s, followed by heating from 40˚C to 75˚C in increments of 0.09˚C/s. Data were analyzed automatically and displayed on the GENECUBE monitor after completion of the assay evaluation.

**Culture and identification of C. difficile**

Briefly, approximately 100 μL stool sample was mixed with 100 μL trypticase soy broth (Becton Dickinson and Company) and treated by ethanol shock for 30 min in an equal volume of 99% ethanol before inoculation, as previously described. Treated stool samples were cultivated with selective agar (CCMA-EX, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 35˚C for 48 h under anaerobic conditions in an anaerobic chamber. Colonies of C. difficile were initially identified by their colony appearance and then confirmed by both matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI/TOF MS, Bruker Corporation, Massachusetts, USA) and the glutamate dehydrogenase (GDH) of the QUIK CHEK.

**Analysis of tcdA, tcdB, cdt genes and tcdC mutation in isolated C. difficile strains**

C. difficile strains were plated on CCMA-EX agar and grown at 35˚C for 46–48 h in anaerobic conditions. Sample preparation was conducted with the concentration of 4 McFarland standard. DNA was extracted from 200 μL of the suspended sample using a QIAamp DNA Mini Kit (QIAGEN N.V., Hilden, Germany) and eluted in a final volume of 100 μL. Real time PCR was performed according to previous papers (Table 1) [22–24].

This assay evaluation was performed on a CFX real-time PCR system (Bio-Rad Laboratories, Inc. Hercules, CA, USA) in a 96-well optical plate format with a THUNDERBIRD Probe qPCR Mix QPS-101 (TOYOBO Co., Ltd.). For testing of the isolates, each 18 μL reaction mixture consisted of 1×THUNDERBIRD Probes, 0.3 μM of each specific primer, 0.2μM of the fluorescent probes, sterile water, and 2 μL DNA template. The thermal cycling conditions were as follows: one cycle 95˚C for 1 min, followed by 40 cycles (45 cycles for testing of stool specimens) at 95˚C for 5 s and 55˚C for 1 min. Date was acquired with the Bio Rad CFX Manager software v 3.0 (Bio Rad).
Investigation of analytical sensitivity and comparison with other assays

For the determination of the limit of detection of the GENECUBE \textit{C. difficile} assay and comparisons with other assays, we used two spiked stool samples and spiked demineralized water for the evaluation. Culture-negative stool samples were pooled and used as matrix of spiked stool samples. \textit{C. difficile} strain ATCC9689 was spiked into negative pooled stool samples at a concentration of $1.5 \times 10^7$ CFU/mL, $1.5 \times 10^6$ CFU/mL, $1.5 \times 10^5$ CFU/mL, $7.5 \times 10^4$ CFU/mL, $5.0 \times 10^4$ CFU/mL, $3.0 \times 10^4$ CFU/mL, $1.5 \times 10^4$ CFU/mL, and $1.5 \times 10^3$ CFU/mL for each set of two pooled stool samples and one demineralized water sample. For the concentration, 1 McFarland was regarded as approximately $3.0 \times 10^7$ CFU/mL as previously described [25]. For the GENECUBE \textit{C. difficile} assay, 50 μL spiked sample was used for assay evaluation and tests were performed four times for each sample. The LODs were estimated as the lowest concentration at which the positivity rate was 100%. As a comparison, the BD MAX Cdiff assay and QUIK CHEK were evaluated with these spiked samples. A single test was performed for each sample. Both the GENECUBE evaluation and the BD MAX evaluation were performed on the same day with the same spiked samples, which were preserved in cool conditions for a day after spiked samples were prepared.

Statistical analyses

The GENECUBE assay results were compared with each result of BD MAX Cdiff assay and toxigenic culture. The positive predictive value and negative predictive value were calculated from routine 2×2 result tables. The 95% confidence intervals (CIs) were calculated by the

Table 1. Primers and probes used for real-time PCR.

| Target genes | Oligonucleotide | Sequence (5’-3’)*b | Amplicon size(bp) | Region | Reference |
|--------------|-----------------|-------------------|-------------------|--------|-----------|
| tcdA         | tcdA_F          | CAGTCGGATTGCAAGTAGTTGCAAT | 102 | A4 | [27] |
|              | tcdA_R          | AGTAGATCTACTACCATTACACGAGTCTGC | | 5891–5993c |
|              | tcdA_P          | FAM-TCGAGATGATACGATGCTCAGGATG-TAMRA |
| tcdB         | tcdB_F          | TACACAACGAGTTGATTTAGTACAGAAGAGATGGA | 240 | [27] |
|              | tcdB_R          | CACCTATTTGATTTAGMCCCTTAAAAAGC | | 5681–5921d |
|              | tcdB_P          | FAM-TGTKCCAGTAAATCAATTGCTT-TAMRA |
| cdtA         | cdtA_F          | GATCTGGTCCTCAAGAATTTGGTT | 103 | A4 | [28] |
|              | cdtA_R          | GCTTGCTTCCATCCATTGATT | | 1051–1153c |
|              | cdtA_P          | FAM-CAAGAGATCTCGTTAGTGACGATATCCAAATTGT-MGBEQ |
| cdtB         | cdtB_F          | AAAAGCTTCTAGTTCTTTTGCACAAG | 132 | A4 | [28] |
|              | cdtB_R          | TATCGAGTAGGCTGATTTTTGATT | | 837–968e |
|              | cdtB_P          | CY5-AACTCTTACTCCTCCCTGAAT-BHQ2 |
| tcdC         | tcdC_F          | GCACAOAGRTATTGCTCTACCTGG | 70 | A4 | [26] |
|              | tcdC_R1         | AGCTGGTAGGATATTTGSCCA |
|              | tcdC_R2         | CAAGATGGTAGGATATTTGSCCA |
|              | tcdC_P_wt       | FAM-AAAACACRCCHAAAATAA-MGBEQ |
|              | tcdC_P_mut      | HEX-AAAACACRCCHAAAATAA-MGBEQ |

*a FAM, 6-carboxylfluorescein; TAMRA, Carboxy tetramethyl-rhodamine; MGBEQ,Minor Groove Binder Eclipse Quencher; CY5, Cy5 carboxylic acid; BHQ-2, Black Hole Quencher 2; HEX, Hexachloro fluorescein.
*b R = A or G; H = A,C or T
*c On the basis of sequence in GeneBank with accession number M30307 for tcdA
*d On the basis of sequence in GeneBank with accession number X53138 for tcdB
*e On the basis of sequence in GeneBank with accession number L76081 for cdtA and cdtB

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Results

Analytical sensitivity

The results of the spike assay evaluation are summarized in Table 2. In the GENECUBE assay evaluation, all positive results (100%) were obtained down to $1.5 \times 10^3$ CFU/mL for demineralized water samples and $3.0 \times 10^4$ CFU/mL for stool samples.

GDH tests were positive down to $5.0 \times 10^4$ CFU/mL for demineralized water samples and stool samples. Molecular assay evaluations of BD MAX were positive down to $1.5 \times 10^4$ CFU/mL for demineralized water samples and $3.0 \times 10^4$ CFU/mL for pooled stool sample 1, however the test was negative for pooled stool sample 2 at the concentrations of $7.5 \times 10^4$ CFU/mL.

Based on these results, the LODs of the GENECUBE assay evaluation were estimated to be $3.0 \times 10^4$ CFU/mL for the detection of toxigenic *C. difficile*.

Clinical stool samples and results of each assay evaluation

A total of 383 clinical stool samples met the study criteria (HUD 106, SMD 29, TUD 21, FUD 17, CTD 80, TRD 54, TCD 39, and TMD 37) and were evaluated by GENECUBE, BD MAX, and culture assay evaluations. In this study, *C. difficile* was cultivated from 85 stool samples and toxin-producing *C. difficile* was cultivated from 60 stool samples (70.6%). Both toxin-producing *C. difficile* and non-toxin producing *C. difficile* were isolated from one stool sample.

Of the 60 toxigenic strains, 55 strains were tcdA-positive/tcdB-positive and five strains were tcdA-negative/ tcdB-positive. cdt mutation was detected in five strains (5/60; 8.3%) and tcdC mutation was detected in one strain (1/60; 1.7%) in this study.
The results of QUIK CHEK showed that GDH was positive in 59/85 *C. difficile* positive stool samples (69.4%) and toxin was positive in 16/60 toxigenic *C. difficile* positive stool samples (26.7%). As for the GENECUBE assay evaluation, positive results were obtained in 55/383 stool samples. No stool samples had the result of “invalid” with the requirement of re-assay evaluation in this study. For the BD MAX assay evaluation, positive results were obtained in 53/383 stool samples. Re-assay evaluation was performed for an invalid result in one stool sample at the first assay evaluation because of high viscosity.

**Comparison of the GENECUBE assay with the BD MAX assay**

The comparison of the GENECUBE assay with the BD MAX assay is summarized in Table 3. The sensitivity, and specificity of the two assays were 99.0% (379/383), 98.1% (52/53) and 99.1% (327/330), respectively.

Of the four stool samples with disconcordance between the two assays, one stool sample was negative by the GENECUBE assay evaluation and positive by the BD MAX assay evaluation. *C. difficile* was not isolated from the stool sample and the GDH assay evaluation of the stool sample was negative. The other three samples were positive by GENECUBE assay evaluation and negative by BD MAX assay evaluation. Toxigenic *C. difficile* was isolated from all three stool samples and all were positive for GDH.

**Comparison of the GENECUBE assay evaluation with toxigenic culture**

The comparison of the GENECUBE assay evaluation with toxigenic culture is summarized in Table 4. The total, sensitivity, and specificity of two assay evaluation were 96.6% (370/383), 85.0% (51/60) and 98.8% (319/323), respectively.

Of the 13 stool samples with disconcordance between the two assays, nine stool samples were negative by the GENECUBE assay evaluation and positive by toxigenic culture. The BD MAX Cdiff assay and GDH assay evaluations were negative in the nine stool samples. The other four samples were positive by the GENECUBE assay evaluation and negative by toxigenic culture. The BD MAX Cdiff assay was positive in the four stool samples and GDH assay evaluation was positive in two of the four stool samples.

Table 3. Comparison of the GENECUBE *Clostridioides difficile* assay with BD MAX Cdiff assay.

|            | GENECUBE         |          | Total (%) |          |
|------------|------------------|----------|-----------|----------|
|            | Positive | Negative |           | 99.0% (97.1–99.4) |
| BD MAX    | 52          | 1        | 53        |          |
| Positive  |            | 327       | 330       |          |
| Negative  | 328        | 383       |          |          |

*GENECUBE, GENECUBE *C. difficile* assay; BD MAX, BD MAX Cdiff assay

a *C. difficile* was not cultivated in selective agar in anaerobic conditions and the GDH assay evaluation of stool sample was negative.

b Toxigenic *C. difficile* was isolated from all three stool samples and GDH assay evaluations of stool samples were all positive.

c Date in parentheses are 95% confidence intervals.

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Discussion

This is the first study evaluating the performance of the GENECUBE C. difficile assay. We report that the assay can be performed without a purification step. Using spiked stool samples and clinical samples, the GENECUBE C. difficile assay detected all GDH-positive toxigenic C. difficile-containing stool samples and had a non-inferior ability to detect the tcdB gene compared with the BD MAX Cdiff assay. In the one case of a negative result by the GENECUBE assay evaluation and positive result by the BD MAX assay evaluation, a false-positive of the BD MAX assay evaluation was considered based on the negative culture result. In the three cases of positive results of the GENECUBE assay evaluation and negative result of the BD MAX assay evaluation, a true-positive was considered based on the positive culture result. There were 13 discordant cases when the results of the GENECUBE C. difficile assay and toxigenic culture were compared in this study. As for the nine cases of negative results of the GENECUBE assay evaluation and positive result of toxigenic culture, we considered that the toxin genes in the stool samples were below the limit of detection of the GENECUBE for the negative result of the BD MAX assay evaluation and GDH assay evaluation. In this study, the four cases were positive results of the GENECUBE assay evaluation and the negative culture result. C. difficile is highly sensitive to the culture method used, especially the alcohol shock procedure [26]. However, even when the alcohol shock procedure was performed, toxic genes were still detected in culture-negative stool samples by molecular examination [27]. We think toxigenic C. difficile was present in the stool samples because positive results were also obtained when using different primers and probes (BD MAX Cdiff assay) for the tcdB gene and when using another detection method (GDH test).

In the previous studies, the sensitivity between molecular assays and toxigenic culture have been reported as 82%–97% for the BD MAX Cdiff assay [7, 10, 15, 28] and 83%–100% for the Xpert C. difficile [7, 10, 15, 28, 29]. Based on these results, we consider that the GENECUBE C. difficile assay has sufficient ability as a molecular assay.

In the clinic, sample-to-answer molecular assay evaluation is useful and two excellent C. difficile assays are commercially available worldwide. The cobas Liat Cdiff assay is the fastest molecular assay for the detection of a toxin gene in C. difficile and is complete in about 20 minutes [8]. The Xpert C. difficile assay requires 45 minutes; however, the assay can detect cdt and tcdC gene mutations in addition to the tcdB gene. Furthermore, the Xpert C. difficile assay is considered to have lowest limit of detection for toxin genes in stool samples [30]. Both assays use a cartridge and do not require laborious preparation procedures. Regarding the

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**Table 4. Comparison of the GENECUBE Clostridioides difficile assay with toxigenic culture.**

| Toxigenic culture | GENECUBE | Total |
|-------------------|----------|-------|
|                   | Positive | Negative | |
| Positive          | 51       | 9a     | 60 |
| Negative          | 4b       | 319     | 323 |
| Total             | 55       | 328     | 383 |

|                  | Total (%) | 96.6% (94.1–98.0) |
|------------------|-----------|------------------|
| Sensitivity (%)  | 85.0% (77.1–89.3) |
| Specificity (%)  | 98.8% (97.3–99.6) |

GENECUBE, GENECUBE C. difficile assay; BD MAX, BD MAX Cdiff assay

a BD MAX Cdiff assay and GDH assay evaluations were negative in the nine stool samples.
b BD MAX Cdiff assay was positive in the four stool samples and GDH assay evaluation was positive in two of the four stool samples.
c Date in parentheses are 95% confidence intervals.

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GENECUBE *C. difficile* assay, the assay evaluation time is as short as for the cobas Liat Cdiff and Xpert *C. difficile* assays, and the GENECUBE *C. difficile* assay is economical because it requires less expensive materials (tips and tubes); however, the preparation and hands-on time are longer than for the cobas Liat Cdiff and Xpert *C. difficile* assays. The GENECUBE system can perform four assays simultaneously and selectively; thus, if the development of assays for the *cdt* gene, *tcdC* mutations, and/or other genes from enteric pathogens are achieved, the GENECUBE *C. difficile* assay will have a higher clinical utility than the current version.

There were a limitation in the current study and the GENECUBE *C. difficile* assay. While assay evaluations of spiked stool samples were performed at same time under the same conditions using the GENECUBE and the BD MAX assays, the BD MAX assay evaluation was performed after the GENECUBE assay evaluation for clinical stool samples and an opposite assay evaluation was not conducted. A delay in BD MAX assay evaluation might negatively affect the test performance of the assay. In addition, current study evaluated the comparison only with the BD MAX assay. Further comparative study such with the Xpert *C. difficile* assay was required for the evaluation of the GENECUBE *C. difficile* assay.

In conclusion, our evaluation indicated that the new non-purification molecular assay has equivalent performance with other current molecular identification assays for *C. difficile* toxin genes.

**Supporting information**

S1 File.

(DOCX)

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References

1. Stanley JD, Bartlett JG, Dart BWt, Ashcraft JH. Clostridium difficile infection. Curr Probl Surg. 2013; 50(7):302–37. Epub 2013/06/15. https://doi.org/10.1067/j.cpsurg.2013.02.004 PMID: 23764949.

2. Dubberke ER, Butler AM, Yokoe DS, Mayer J, Hota B, Mangino JE, et al. Multicenter study of Clostridium difficile infection rates from 2000 to 2006. Infect Control Hosp Epidemiol. 2010; 31(10):1030–7. Epub 2010/08/11. https://doi.org/10.1086/656245 PMID: 20695799; PubMed Central PMCID: PMC3648217.

3. Davies K, Davis G, Barbut F, Eckert C, Petrosillo N, Wilcox MH. Variability in testing policies and impact on reported Clostridium difficile infection rates: results from the pilot Longitudinal European Clostridium difficile Infection Diagnosis surveillance study (LuCID). Eur J Clin Microbiol Infect Dis. 2016; 35(12):1949–56. Epub 2016/09/04. https://doi.org/10.1007/s10096-016-2746-1 PMID: 27590621; PubMed Central PMCID: PMC5138271.

4. Kato H, Senoh M, Honda H, Fukuda T, Tagashira Y, Horuchi H, et al. Clostridiodes (Clostridium) difficile infection burden in Japan: A multicenter prospective study. Anaerobe. 2019. Epub 2019/03/16. https://doi.org/10.1016/j.anaerobe.2019.03.007 PMID: 30872073.

5. Riley TV, Kimura T. The Epidemiology of Clostridium difficile Infection in Japan: A Systematic Review. Infect Dis Ther. 2018; 7(1):39–70. Epub 2018/02/15. https://doi.org/10.1007/s40121-018-0186-1 PMID: 29441500; PubMed Central PMCID: PMC5840105.

6. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, et al. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis. 2018; 66(7):987–94. Epub 2018/03/22. https://doi.org/10.1093/cid/ciy149 PMID: 29562266.

7. Shin BM, Yoo SM, Shin WC. Evaluation of Xpert C. difficile, BD MAX Cdiff, IMdx C. difficile for Abbott m2000, and Illumigene C. difficile Assays for Direct Detection of Toxigenic Clostridium difficile in Stool Specimens. Ann Lab Med. 2016; 36(2):131–7. Epub 2015/12/29. https://doi.org/10.3343/alm.2016.36.2.131 PMID: 26702620; PubMed Central PMCID: PMC4713846.

8. Hethem DJ, Bos-Sanders I, Nijhuis RHT, Tammenga S, Berlinger L, Kuiper EJ, et al. Evaluation of the Liat Cdiff Assay for Direct Detection of Clostridiodes difficile Toxin Genes within 20 Minutes. J Clin
Ito Y, Iwashima S, Hayano S, Nishio T, Shiozawa R, Yata S, et al. Rapid Detection of the Macrolide

Akashi Y, Hayashi D, Suzuki H, Shiigai M, Kanemoto K, Notake S, et al. Clinical features and seasonal

17. Hida Y, Hisada K, Shimada A, Yamashita M, Kimura H, Yoshida H, et al. Rapid detection of the

10.1016/j.diagmicrobio.2014.06.001 PMID: 25027069.

11. Hanne I, Engshbro AL, Pareja J, Schneider UV, Lisby JG, Pruzinec-Popovic B, et al. Multicenter evaluation
of the new QIAstat Gastrointestinal Panel for the rapid syndromic testing of acute gastroenteritis. Eur J Clin Microbiol Infect Dis. 2019. Epub 2019/07/29. https://doi.org/10.1007/s10096-019-03646-4 PMID: 31352670.

12. Leal SM Jr., Popowitch EB, Levinson KJ, John TM, Lehman B, Rios MB, et al. Quantitative Thresholds Enable Accurate Identification of Clostridium difficile Infection by the Luminox xTAG Gastrointestinal Pathogen Panel. J Clin Microbiol. 2018; 56(6), Epub 2018/04/13. https://doi.org/10.1128/JCM.01885-17 PMID: 29643194; Pubmed Central PMID: PMC5971534.

13. Babady NE, Stiles J, Ruggiero P, Khosar P, Huang D, Shupert S, et al. Evaluation of the Cepheid Xpert Clostridium difficile Epi assay for diagnosis of Clostridium difficile infection and typing of the NAP1 strain at a cancer hospital. J Clin Microbiol. 2010; 48(12):4519–24. Epub 2010/10/15. https://doi.org/10.1128/JCM.01648-10 PMID: 20943860; Pubmed Central PMID: PMC3008447.

14. Jamal W, Pauline EM, Rotimi VO. Comparative performance of the GeneXpert C. difficile PCR assay and C. difficile Quik Chek Complete kit assay for detection of Clostridium difficile antigen and toxins in symptomatic community-onset infections. Int J Infect Dis. 2014; 29:244–8. Epub 2014/12/03. https://doi.org/10.1016/j.ijid.2014.10.025 PMID: 25462186.

15. Yoo J, Lee H, Park KG, Lee GD, Park YJ. Evaluation of 3 automated real-time PCR (Xpert C. difficile assay, BD MAX Cdiff, and IDx C. difficile for Abbott m2000 assay) for detecting Clostridium difficile toxin gene compared to toxigenic culture in stool specimens. Diagn Microbiol Infect Dis. 2015; 83(1):7–10. Epub 2015/06/18. https://doi.org/10.1016/j.diagmicrobio.2015.05.005 PMID: 26081240.

16. Hida Y, Hisada K, Shimada A, Yamashita M, Kimura H, Yoshida H, et al. Rapid detection of the Mycobacterium tuberculosis complex by use of quenching probe PCR (geneCube). J Clin Microbiol. 2012; 50(11):3604–8. Epub 2012/08/31. https://doi.org/10.1128/JCM.01654-12 PMID: 22933602; Pubmed Central PMID: PMC3486240.

17. Miyazaki N, Yamagishi Y, Izumi K, Kawashima Y, Suematsu H, Mikamo H. Evaluation of rapid measurement of Chlamydia trachomatis and Neisseria gonorrhoeae by using automatic gene analyzer "GENECUBE". Jpn J Antibiot. 2016; 69(4):291–8. Epub 2016/08/01. PMID: 30226955.

18. Akashi Y, Hayashi D, Suzuki H, Shigai M, Kanemoto K, Notake S, et al. Clinical features and seasonal variations in the prevalence of macrolide-resistant Mycoplasma pneumoniae. J Gen Fam Med. 2018; 19(6):191–7. Epub 2018/11/23. https://doi.org/10.1002/jgf2.201 PMID: 30464865; PubMed Central PMCID: PMC6238234.

19. Ito Y, Iwashima S, Hayano S, Nishio T, Shiozawa R, Yata S, et al. Rapid Detection of the Macrolide Sensitivity of Pneumonia-Causing Mycoplasma pneumoniae Using Quenching Probe Polymerase Chain Reaction (GENECUBEx). Mol Diagn Ther. 2018; 22(6):737–47. Epub 2018/09/28. https://doi.org/10.1007/s40291-018-0360-x PMID: 30259422.

20. Hida Y, Uemura K, Sugimoto H, Kawashima Y, Koyanagi N, Notake S, et al. Evaluation of performance of the GENECUBE assay for rapid molecular identification of Staphylococcus aureus and methicillin resistance in positive blood culture medium. PLoS One. 2019; 14(7):e0219819. Epub 2019/07/17. https://doi.org/10.1371/journal.pone.0219819 PMID: 31310615; PubMed Central PMID: PMC6634395.

21. Blake MR, Raker JM, Whelan K. Validity and reliability of the Bristol Stool Form Scale in healthy adults and patients with diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther. 2016; 44(7):693–703. Epub 2016/08/06. https://doi.org/10.1111/apt.13746 PMID: 27492648.

22. de Boer RF, Wijma JJ, Schuurman T, Moedt J, Dijk-Alberts BG, Ott A, et al. Evaluation of a rapid molecular screening approach for the detection of toxigenic Clostridium difficile in general and subsequent identification of the tcdC Delta117 mutation in human stools. J Microbiol Methods. 2010; 83(1):59–65. Epub 2010/08/03. https://doi.org/10.1016/j.mimet.2010.07.017 PMID: 20674616.

23. Kubota H, Sakai T, Gawad A, Makino H, Akiyama T, Ishikawa E, et al. Development of TaqMan-based quantitative PCR for sensitive and selective detection of toxigenic Clostridium difficile in human stools. PLoS One. 2014; 9(10):e111684. Epub 2014/11/02. https://doi.org/10.1371/journal.pone.0111684 PMID: 25360662; PubMed Central PMID: PMC4216139.
24. Wroblewski D, Hannett GE, Bopp DJ, Dumyati GK, Halse TA, Dumas NB, et al. Rapid molecular characterization of *Clostridium difficile* and assessment of populations of *C. difficile* in stool specimens. J Clin Microbiol. 2009; 47(7):2142–8. Epub 2009/05/01. https://doi.org/10.1128/JCM.02498-08 PMID: 19403775; PubMed Central PMCID: PMC2708487.

25. Mashock MJ, Faron ML, Buchan BW, Ledeboer NA. Evaluation of Copan FecalSwab as Specimen Type for Use in Xpert *C. difficile* Assay. J Clin Microbiol. 2017; 55(10):3123–9. Epub 2017/08/11. https://doi.org/10.1128/JCM.00369-17 PMID: 28794179; PubMed Central PMCID: PMC5625397.

26. Clabots CR, Gerding SJ, Olson MM, Peterson LR, Gerding DN. Detection of asymptomatic *Clostridium difficile* carriage by an alcohol shock procedure. J Clin Microbiol. 1989; 27(10):2386–7. Epub 1989/10/01. PMID: 2685035; PubMed Central PMCID: PMC267032.

27. Kato H, Yokoyama T, Kato H, Arakawa Y. Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. J Clin Microbiol. 2005; 43(12):6108–12. Epub 2005/12/08. https://doi.org/10.1128/JCM.43.12.6108-6112.2005 PMID: 16333105; PubMed Central PMCID: PMC1317188.

28. Chiang D, Ng S, La MV, Jureen R, Lin RT, Teo JW. Performance assessment of the BD MAX Cdiff assay in comparison to Xpert *C. difficile* assay in a setting with very low prevalence of toxigenic *Clostridium difficile* PCR ribotype 027. Anaerobe. 2014; 30:156–8. Epub 2014/10/12. https://doi.org/10.1016/j.anaerobe.2014.09.021 PMID: 25305151.

29. Kraft CS, Parrott JS, Cornish NE, Rubinstein ML, Weissfeld AS, McNult P, et al. A Laboratory Medicine Best Practices Systematic Review and Meta-analysis of Nucleic Acid Amplification Tests (NAATs) and Algorithms Including NAATs for the Diagnosis of *Clostridioides* (*Clostridium*) *difficile* in Adults. Clin Microbiol Rev. 2019; 32(3). Epub 2019/05/31. https://doi.org/10.1128/cmrr.00032-18 PMID: 31142497; PubMed Central PMCID: PMC6589859.

30. Gyorke CE, Wang S, Leslie JL, Cohen SH, Solnick JV, Polage CR. Evaluation of *Clostridium difficile* fecal load and limit of detection during a prospective comparison of two molecular tests, the illumigene *C. difficile* and Xpert *C. difficile/Epi* tests. J Clin Microbiol. 2013; 51(1):278–80. Epub 2012/10/12. https://doi.org/10.1128/JCM.02120-12 PMID: 23052320; PubMed Central PMCID: PMC3536201.