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RESEARCH ARTICLE

Accuracy of loop-mediated isothermal amplification for the diagnosis of *Clostridium difficile* infection: a systematic review

Aaron Lloyd¹, Vinay Pasupuleti¹, Priyaleela Thota¹, Chaitanya Pant², David D.K. Rolston³, Adrian V. Hernandez^{4,5}, Vicente A. Benites-Zapata⁶, Thomas G. Fraser⁷, Curtis J. Donskey^{1,8}, Abhishek Deshpande^{7,9}

1) *Department of Medicine, School of Medicine, Case Western Reserve University, Cleveland, OH*, 2) *Department of Gastroenterology, University of Kansas Medical Center, Kansas City, KS*, 3) *Department of Internal Medicine, Geisinger Medical Center, Danville, PA, USA*, 4) *Health Outcomes and Clinical Epidemiology Section, Department of Quantitative Health Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA*, 5) *Postgraduate and Medical Schools, Universidad Peruana de Ciencias Aplicadas (UPC)*, 6) *Faculty of Human Medicine, Postgraduate Section, Universidad de San Martin de Porres, Lima, Peru*, 7) *Department of Infectious Diseases, Medicine Institute, Cleveland Clinic*, 8) *Geriatric Research Education and Clinical Center, Cleveland VA Medical Center*, 9) *Medicine Institute Center for Value Based Care Research, Cleveland Clinic, Cleveland, OH, USA*

AL and VP contributed equally to this manuscript.

Running title: LAMP assay for diagnosis of *C. difficile* infection

Corresponding author: Abhishek Deshpande MD, PhD, Medicine Institute Center for Value Based Care, Cleveland Clinic, 9500 Euclid Avenue, Desk G1-40, Cleveland OH 44195, USA. Email: abhishekdp@gmail.com

Highlights: Nucleic acid amplification tests are currently used as standalone tests for the diagnosis of CDI. However current IDSA/SHEA guidelines recommend more data before routine use. LAMP appears to be highly sensitive and specific method for the rapid diagnosis of CDI.

Abstract

Loop-mediated isothermal DNA amplification (LAMP) are currently used as standalone diagnostic test for *C. difficile* infection (CDI). We assessed the diagnostic accuracy of LAMP for the diagnosis of CDI. We searched 5 databases to identify studies that compared LAMP with culture cytotoxicity neutralization assay or anaerobic toxigenic culture (TC) of *C. difficile*. We used the random-effects model to calculate pooled sensitivities, specificities, diagnostic odds ratios and their 95% confidence intervals (CIs). The search of the databases yielded 16 studies (6,979 samples) that met inclusion criteria. When TC was used as the gold standard (6,572 samples), bivariate analysis yielded a mean sensitivity of 0.95 (95%CI, 0.93-0.97; $I^2 = 67.4$) and a mean specificity of 0.99 (95%CI, 0.96-1.00; $I^2 = 97.0$). LAMP is a useful diagnostic tool with high sensitivity and specificity for detecting CDI. The results should however be interpreted only in the presence of clinical suspicion and symptoms of CDI.

Introduction

Clostridium difficile is the most common bacterial cause of in-hospital healthcare-associated diarrhea in North America and Europe with an estimated incidence of 3.85 cases per 1000 patient-days in US acute care hospitals [1]. Over the last several years, new, highly virulent strains, such as BI-NAP1-027, have caused several global outbreaks [2]. These strains have been shown to cause more severe disease than the non-NAP1 strains and are associated with high level fluoroquinolone resistance [2-4]. In addition to the associated morbidity and mortality, the cost of hospital-acquired *Clostridium difficile* infections (CDI) has been estimated to exceed \$1.5 billion a year in the United States [1]. Nearly all antimicrobials have been associated with CDI over the years, with longer exposure and exposure to multiple antimicrobial agents increasing the risk of CDI further [5]. The diagnosis of CDI is usually made based on the presence of symptoms (diarrhoea, abdominal pain, fever) and either a stool test result positive for *C. difficile* toxins or toxigenic *C. difficile*, or colonoscopic findings demonstrating pseudomembranous colitis [6]. Laboratory test methods available for identification of CDI include anaerobic toxigenic culture (TC), culture cytotoxicity neutralization assay (CCNA), enzyme immunoassay (EIA) for toxin A and B, glutamate dehydrogenase (GDH) EIA, real-time polymerase chain reaction (RT-PCR), and Loop-mediated Isothermal Amplification assay (LAMP) [7]. There is currently no accepted gold standard test for diagnosis of CDI, but CCNA (sensitivity of 70-100%, specificity of 90-100%) and anaerobic TC (sensitivity of 90-100%, specificity of 98-100%) are often used as reference tests for evaluation of each other as well as evaluation of novel testing methods [8,9]. CCNA and anaerobic TC are time-consuming and resource intensive

tests. In practice, many labs perform the rapid and easy to perform EIA for toxin A and B detection, though this test lacks sensitivity and is considered a suboptimal approach by current clinical practice guidelines [6]. More recently, nucleic acid amplification tests including real-time PCR and LAMP have been developed for diagnosis of CDI. Some hospitals have already begun to implement these tests in order to improve the rapidity of CDI testing and detection rates. Two previous meta-analyses have evaluated at the diagnostic characteristics of real-time PCR. They found that while it is highly sensitive and specific, it is also dependent on CDI prevalence [7,10]. While most commercially available real-time PCR assays are designed to detect a conservative region of *tcdB*, it has been reported that in *C. difficile* variant strains, *tcdA* is more conserved [11]. The Illumigene™ *C. difficile* Assay (Meridian Bioscience, Cincinnati, OH) uses loop-mediated isothermal amplification technology to detect a 204-bp sequence in the conserved 5' region of *tcdA*. While some variant *C. difficile* strains have deletions at the 3' end of the *tcdA* gene, the 5' portion remains intact for these strains [12]. A recent study confirmed the ability of the Illumigene assay to detect these Toxin A-/B+ strains [13]. The Illumigene assay is currently the only commercial LAMP assay approved for U.S. laboratory use. The FDA now requires all vendors to include toxigenic culture as a comparator for any new diagnostic test for *C. difficile* detection. One systematic review evaluated the diagnostic capabilities of LAMP for detection of CDI and concluded that LAMP was a promising test, but further investigation was necessary to evaluate LAMP as a diagnostic tool [7]. The most recent Infectious Diseases Society of America (IDSA)/Society for Healthcare of America (SHEA) guidelines on diagnostic testing of *C. difficile* suggest that more data are needed on nucleic acid amplification tests before it

can be implemented for routine use [6]. We performed a meta-analysis to assess the capabilities of LAMP in the diagnosis of CDI. The aim of this study was to investigate whether LAMP is sensitive and specific enough for the diagnosis of CDI when used as a stand-alone test.

Methods

This review was performed with a standardized written protocol that followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement guidelines [14].

Search Strategy

We systematically searched the literature using the following predetermined inclusion criteria. Studies evaluating LAMP as a diagnostic test for CDI were eligible for inclusion if the studies (1) described original research; (2) performed stool specimen analyses from inpatients or outpatients; (3) compared LAMP to a reference method - either CCNA or anaerobic TC; (4) reported total number of patients tested and positive/negative results that allow calculation of true positives (TP), true negatives (TN), false positives (FP), false negatives (FN); (5) in any language. We excluded studies if (1) all samples were not tested by at least 1 reference test, that is, CCNA or anaerobic TC; (2) if the reference test was performed only on a subset of samples, that is, only positives, negatives, or those that were discordant; (3) the reference test was a combination of >1 diagnostic test and (4) they involved animal studies or laboratory cultures of *C. difficile*. In the excluded studies, LAMP tests were confirmed using other diagnostic tests but not

CCNA or anaerobic TC. We also excluded combination reference tests because we wanted to individually calculate the diagnostic accuracy of LAMP vis-a`-vis the 2 most accepted reference standards. This search was performed in August 2014. The following databases were searched since their inception: PubMed, Web of Science, Embase, Cochrane Library, and Scopus. In addition, we reviewed the reference lists of several previously published reviews on *C. difficile* diagnostic testing. Search terms included “*Clostridium difficile*, *C. difficile*, CDI, sensitivity, specificity, screening, false positives, false negatives, accuracy, predictive value, diagnostic test, diagnosis.” Reference lists from included studies were also searched. In addition, experts and commercial LAMP assay manufacturer for *C. difficile* diagnosis were contacted for additional studies. The electronic search strategy of PubMed is available in Supplementary Appendix 1.

Study Selection

A list of retrieved articles were reviewed independently by 2 investigators (A. L. and V. P.) in order to choose potentially relevant articles, and disagreements about particular studies were discussed and resolved. When multiple articles for a single study had been published by the same authors, we used the most relevant publication and supplemented it, if necessary, with data from other publications. Authors of studies were contacted when the information was not available in the published study.

Data Extraction

Two investigators (A. L. and V. P.) independently extracted data from full text of the included studies, and disagreements were discussed and resolved. All studies evaluated the diagnostic accuracy of LAMP on a per-sample basis.

Assessment of Study Quality

The methodological quality for each paper was assessed independently by 2 investigators (A. L. and V. P.) using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) [15] criteria and disagreements were discussed and resolved.

Data Synthesis and Meta-Analysis

The TP, FP, TN, and FN were taken directly from the source papers. Where this information was not available, the values were calculated from the data that were provided in the article. In some cases the corresponding authors of the article were contacted to gather information for creating the 2 X 2 table. Data were analysed using the 'midas' and 'metandi' module for STATA (version 12) and two freeware programs i.e. Open Meta[analyst] (version 1.4) [16] and Meta-disc (MetaDiSc (version 1.4). To synthesize data, we employed a bivariate random-effects model. The bivariate model pairs sensitivity and specificity simultaneously for each study, accommodating their natural correlation, thus preserving the two-dimensional nature of the data and allows for negative correlation within studies [17]. Using a random-effects approach for both sensitivity and specificity the model also accounts for clinical and methodological heterogeneity between the studies [18]. We also calculated the positive likelihood ratio (PLR), negative likelihood ratio (NLR), and a hierarchical summary receiver-operating

characteristics (HSROC) curve. To address 0 observations in 2×2 contingency tables, continuity correction was achieved by adding 0.5 to each cell. We calculated κ statistics to assess the agreement between the two investigators for study selection, data extraction and assessment of methodological quality.

Investigations of Heterogeneity

To assess heterogeneity, we calculated the inconsistency index, I^2 . An I^2 of 33-66% was considered as moderate heterogeneity. To address potential heterogeneity among studies, we performed subgroup analysis on pre-specified variable: the calculated prevalence of *C. difficile* (<15% and $\geq 15\%$). As different cut-offs or thresholds were not expected for the LAMP assay among the studies, we did not explore threshold effect as a potential source of heterogeneity.

Publication Bias

The presence and effect of publication bias were examined using Deeks' regression test of asymmetry [19] and Egger's test [20].

Fagan's nomogram

The relationship between pre-test probability and post-test probability was depicted by visual Fagan's nomogram [21]. The pre-test and post-test probabilities are both subjective estimates of the presence of a disease before and after a diagnostic test.

Results

Study Characteristics

Our search yielded 60 articles; 30 potentially relevant citations were selected based on relevance to the study topic. After reviewing the titles and abstracts, 21 articles were selected for full-text evaluation (Figure 1). Sixteen articles with 18 studies published between 2005 and 2014 reported the sensitivity and specificity of LAMP on stool samples for the diagnosis of *C. difficile* infection, and were included in our meta-analysis [22-37]. Five articles were excluded (reasons for exclusion in Figure 1). The inter-rater reliability for study selection was 0.96.

Table 1 summarizes the main study characteristics of the included studies. In total, 6,979 stool samples from patients at risk of CDI were included in the meta-analysis. The prevalence of CDI across all studies ranged from 10% to 60%. LAMP was used for diagnostic purposes in all the studies and not for screening patients. The quality of studies as assessed by the QUADAS-2 tool was generally high, with all but one study [35] meeting 6 or more of the criteria (Supplementary figure 1A and 1B). Three studies [28,36,37] used CCNA as a reference test. In one study [28], the investigators reported the diagnostic accuracy separately for both the reference standards. The inter-rater reliability for assessment of quality items was 0.88.

Meta-Analysis

Results are given as values (95% CI). With anaerobic TC as the reference test (15 studies, 6,572 samples) and using a bivariate random-effects model the results were as

follows: sensitivity 0.95 (0.93–0.97) $I^2 = 67.4\%$; specificity 0.99 (0.96–1.00) $I^2 = 97.0\%$ (Figure 2); PLR 70.29 (25.26–195.55) $I^2 = 94.0\%$; NLR 0.05 (0.03–0.08), $I^2 = 85.0\%$; DOR 1459.4 (611.87–3480.923), $I^2 = 94.0\%$; and AUC 0.99 (.97–.99) (Figure 3). There was substantial heterogeneity for all the statistical measures.

The statistically non-significant p-value for the slope co-efficient suggests symmetry in the data and a relative low likelihood of publication bias by Deeks' regression test of asymmetry ($t = -1.56$; $p = 0.14$) (Figure 4). The relationship between pre-test probability and post-test probability was depicted by visual Fagan's nomogram. For patients with a pre-test probability of 20%, the post-test probability of positive results was 95% and post-test probability of negative results was 1% (Figure 5).

As there were less than 4 studies with CCNA as the reference standard, we could not undertake a bivariate analysis or fit a hierarchical model with all 5 parameters by maximum likelihood. Therefore, a separate univariate meta-analysis was performed. With CCNA as the reference test (3 studies, 407 samples), the results were as follows: sensitivity was 0.93 (0.85–0.97), $I^2 = 68.6\%$; specificity 0.91 (0.87–0.94), $I^2 = 90.7\%$; PLR 9.30 (2.87–30.14), $I^2 = 90.3\%$; NLR 0.12 (0.06–0.24), $I^2 = 0\%$; DOR 123.90 (46.76–328.29), $I^2 = 0\%$; and AUC 0.97 (.94–1.00) (Supplemental figures 2A – 2F). Publication bias was not evaluated as there were inadequate number of included studies (<10) to properly assess a funnel plot.

We explored the source of heterogeneity in terms of CDI prevalence and sample size. Analysis stratified by region was unpractical because of ≤ 3 studies from each country.

Subgroup Analysis by CDI Prevalence

Using a bivariate random-effects model, at a prevalence of <15% (5 studies, 2,576 samples) the sensitivity was 0.90 (0.84–0.94), $I^2 = 39.43\%$; specificity 1.00 (0.99–1.00), $I^2 = 35.98\%$; DOR 2876.12 (1168.35–7080.533) $I^2 = 0\%$. The calculated predictive values at a prevalence of <15% were: PPV 1.00 (1.00–1.00) and NPV 0.99 (0.98–0.99). Using a bivariate random-effects model, at a prevalence between $\geq 15\%$ (10 studies, 3,996 samples) the sensitivity was 0.96 (0.94–0.98), $I^2 = 34.72\%$; specificity 0.97 (CI: 0.91–0.99), $I^2 = 95.8\%$; DOR 748.05 (254.43–21299.31), $I^2 = 70\%$. The calculated predictive values at a prevalence of <15% were: PPV 0.94 (0.84–0.98), NPV 0.98 (0.97–0.99).

Subgroup Analysis by Sample Size

The median sample size was 272. In studies with a sample size < 272 (7 studies, 767 samples) the sensitivity was 0.93 (0.88–0.96), $I^2 = 38.54\%$; specificity 0.98 (0.85–1.00), $I^2 = 91.80\%$; PLR 50.72 (5.85–439.57) $I^2 = 80.5\%$; NLR 0.07 (0.04–0.12), $I^2 = 0\%$; DOR 729.98 (86.27–6176.92) $I^2 = 42.6\%$.

In studies with a sample size ≥ 272 (8 studies, 5,805 samples) the sensitivity was 0.96 (0.92–0.98), $I^2 = 75.2\%$; specificity 0.99 (0.97–1.00), $I^2 = 95.9\%$; PLR 87.21 (32.53–233.77) $I^2 = 96\%$; NLR 0.04 (0.02–0.09), $I^2 = 88.6\%$; DOR 2069.2 (1176.11–3640.46), $I^2 = 0\%$.

With anaerobic TC as the reference test, theoretical values of PPV and NPV were calculated using the pooled sensitivity (0.95) and specificity (0.99) values and plotted

against increasing CDI prevalence. The PPV values correlated positively with increasing CDI prevalence, whereas NPV remained almost the same (Figure 6).

In studies with anaerobic TC as the reference test, sensitivity analysis was also performed by omitting studies individually from the meta-analysis, and the pooled results of the remaining studies were largely the same (data not shown).

Discussion

The LAMP assay evaluated here, which amplifies the *C. difficile* *tcdA* gene, demonstrated high sensitivity and specificity in comparison to anaerobic TC as the reference standard for diagnosing CDI. Despite statistical heterogeneity, the diagnostic accuracy was consistently similar in the direction of effect in the majority of the studies. The likelihood ratios for the LAMP assay indicate that the test is useful in determining post-test probability of CDI. Also, the PPV increases as the prevalence increases and reaches 95%, for a CDI prevalence of $\geq 15\%$.

The rising incidence and severity of CDI continues to be a major challenge for health-care institutions. A rapid, accurate diagnosis followed by prompt treatment are critical in the management of CDI and prevention of transmission [38,39]. Barbut *et al.* found that a change from toxigenic culture and CTA to a NAAT or a 2-step algorithm including GDH and NAAT resulted in a significant reduction in the time to reporting of test results and earlier initiation of treatment, reduced empiric therapy in patients without CDI, and a decrease in processing of multiple samples. The authors concluded that

reducing delays in testing may improve the quality of patient management [40]. However, as with real-time PCR, the diagnosis of CDI should only be made in the presence of a strong clinical suspicion and symptoms consistent with CDI. A positive LAMP test result can only identify the presence of the *tcdA* gene in the feces and thus the clinical assessment remains essential to detect true infection. Molecular methods like LAMP have a quick turnaround time of less than 4 hours and therefore are helpful for timely implementation of infection control measures.

Several recent studies have demonstrated that real-time PCR for toxin B gene has a high sensitivity and specificity in detecting CDI [7,10]. In a recent diagnostic accuracy meta-analysis evaluating real-time PCR (toxin B gene) the pooled sensitivity and specificity were 92% and 94%, respectively. In contrast, our meta-analysis found a pooled sensitivity and specificity of LAMP to be 95% and 99% respectively. These data suggest that possibility that the LAMP assay may be more sensitive and specific than real-time PCR in detecting CDI. Also, the PPV and NPV for LAMP are better than real-time PCR in settings where CDI prevalence is <15% [7]. Therefore, real-time PCR may be more suitable in epidemic conditions with higher *C. difficile* prevalence and LAMP may be better in settings with lower *C. difficile* prevalence. In a previous systematic review of 6 studies published in 2012, O'Horo *et al*, evaluated the performance of LAMP in the detection of CDI [7]. They concluded that LAMP was a promising test and further investigation was necessary to evaluate LAMP as a diagnostic tool. Our systematic review identified several additional studies since the publication of their review and used the bivariate random-effects model to pool estimates.

Our findings indicate that LAMP is a useful diagnostic test with a high sensitivity, specificity, likelihood ratios and post-test probability. Given a pre-test probability of 20%, the post-test probability for a positive test results was 95% and post-test probability of negative test result was 1%. There are however some potential limitations of using LAMP assay as a diagnostic tool. The LAMP assay very specifically detects the *tcdA* gene encoding the toxin and not the toxin itself. Therefore, patients with asymptomatic colonization with *C. difficile* can be misdiagnosed as true positives if inappropriate testing is performed. Additional studies are needed to better understand the role of LAMP for detection of asymptomatic colonization. Also, the test may remain positive for several weeks after the resolution of clinical symptoms and should not be used as a test for cure. Lastly, while the majority of clinicians agree that asymptomatic carriers should not be treated; there is no consensus on whether infection control interventions should be directed at isolation of the asymptomatic carriers.

Strengths and limitations of the review

We used a comprehensive search strategy to identify all relevant studies. Our search involved five unique and large databases with no language restrictions. Also, there was relatively low likelihood of publication bias as assessed by Deek's method.

The present study has some limitations. First, the varied study designs and the substantial heterogeneity among the pooled estimates soften any conclusions drawn from our meta-analysis. To minimize this, only studies that used TC or CCNA as the reference standard were included and analysed separately. We anticipated some

degree of heterogeneity across the studies and performed pre-specified subgroup analyses (CDI prevalence and sample size) to reduce potential heterogeneity. None of the study characteristics however, were responsible for the majority of this heterogeneity. It is possible that hidden, unrecorded differences may have contributed to the heterogeneity. Second, our meta-analysis could not adjust for differences in patient populations, study variables and institutional characteristics. For example, prescription of the test must be strictly limited to patients with diarrhoea to avoid detecting asymptomatic carriers. Unfortunately, most studies did not specify the exact criteria used to submit patient stool samples for testing and we do not know definitively whether all patients with symptomatic diarrhoea were having CDI.

Conclusions

LAMP assay appears to be a highly sensitive and specific method for the rapid diagnosis of CDI. However as a relatively newer assay, and considerable heterogeneity among studies, a cautious approach may be needed, with more large scale, well designed studies to assess its true potential.

Reporting

The study was reported according to the PRISMA Guidelines.

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Conflict of Interest

All authors report no conflict of interest.

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Table 1. Basic characteristics of included studies

First author	Year published	Study location	Study years	Sample size	Reference test	Calculated prevalence (%)
Bruins MJ	2012	Netherlands	2010-2011	982	Anaerobic TC	14.8
Buchan BW	2012	USA	NA	96	Anaerobic TC	15.6
Calderaro A	2013	Italy	2011	306	Anaerobic TC	19.6
Dubberke E	2011	USA	2010	150	Anaerobic TC	29.3
Floré K	2012	Belgium	2010-2011	185	Anaerobic TC	10.8
Floré K - 2	2012	Belgium	NA	30	Anaerobic TC	60.0
Gyorke CE	2012	USA	2011	799	Anaerobic TC	14.6
Kato H	2005	Japan	NA	74	Anaerobic TC	54.1
Lalande V	2011	France	NA	472	Anaerobic TC	10.4
Norén T	2011	Sweden	2010	272	Anaerobic TC	18.4
Norén T	2012	Sweden	2010-2011	2242	Anaerobic TC	16.1
Norén T	2014	Sweden	2011	302	Anaerobic TC	29.1
Viala C	2012	France	2009-2010	94	Anaerobic TC	47.9
Ylisiurua P	2013	Finland	2010-2011	430	Anaerobic TC	40.9
Zidaric V	2011	Slovenia	2010	138	Anaerobic TC	13.8
Kato H	2005	Japan	NA	67	CCNA	47.8
Ota KV	2012	USA	2011	141	CCNA	18.4
Pancholi P	2012	USA	2010	199	CCNA	16.9

NA = not available; TC = toxigenic culture; CCNA = cell cytotoxicity neutralization assay

Figure Legend:

Figure 1: Flowchart of Literature Search and Disposition of Articles Screened for Inclusion

Figure 2: Sensitivity and Specificity of Loop-mediated isothermal amplification assay for diagnosis of *C. difficile* infection using anaerobic toxigenic culture as the reference test. The sensitivity and specificity are represented by individual squares and the horizontal lines represent the 95% confidence intervals for each included study. The diamonds represent the pooled summary estimates (95% CI).

Figure 3: Hierarchical summary receiver operating characteristic (HSROC) curve for the diagnosis of *C. difficile* infection. Each circle represents a study. The size of the circle is proportional to the weight given to the study in the final accuracy estimate. The marked point on the curve represents the summary sensitivity and specificity. The area delimited by dashed line represents 95% confidence interval of the summary estimate. The area delimited by the dots represents the 95% prediction region, within which there is a 95% confidence that the true sensitivity and specificity of a future study should lie.

Figure 4: Deeks' funnel plot asymmetry test for publication bias.

Figure 5: Fagan nomogram of Loop-mediated isothermal amplification assay for diagnosis of *C. difficile* infection using anaerobic toxigenic culture as the reference test

Figure 6: Theoretical values of positive predictive value and negative predictive value for increasing *C. difficile* infection prevalence calculated using pooled sensitivity (95%) and specificity (99%).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6