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Critically Appraised Topic

Clostridioides difficile diagnosis

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Clinical Bottom Line

Clostridioides (formerly known as "*Clostridium*") *difficile* is an anaerobic, sporulating, gram-positive bacteria, capable of causing severe diarrheal disease due to the secretion of potent toxins. It is an important cause of healthcare-associated infections and the complications, extended duration of hospitalization, isolation measures, laboratory investigations and treatments account for a significant clinical and economic burden. A rapid and accurate diagnosis is crucial in initiating treatment and preventing complications for any single patient, as well as in enabling isolation precautions to avoid further spreading of spores. While there have been many advances in diagnostic tests of *C. difficile*, the 'optimal' testing strategy remains a matter of some controversy.

A literature search was performed to characterize the diagnostic performances of various assays used in the diagnosis of *C. difficile.* Additionally, several renowned guidelines were consulted to evaluate the current diagnostic flow of the microbiology laboratory of the University Hospitals Leuven (UZL). Information on requests dating between 01/01/2010 and 31/12/2021 was extracted from the laboratory system and used to evaluate requesting habits.

The diagnostic algorithm used is in accordance with most international guidelines. Test utilization appears stable. Many repeat requests occur within 7 days (38%; 824/2180). Strikingly few cases are detected by simultaneous measurement of GDH and toxin. Optimization of the current diagnostic flow is possible: (1) by enforcing the standard operating protocols in place; (2) by changing the diagnostic algorithm and (3) by implementing more restrictive requesting rules.

Clinical/Diagnostic Scenario

Introduction

Clostridioides difficile was first described in 1935 as a potentially pathogenic anaerobic bacteria isolated from the first passage stool specimens of healthy new-born infants. Somewhat ironically, the species name reflects the difficulty the researchers faced with its culture and isolation. Already in this very first paper, the presence of germinating spores and potent soluble exotoxins was described.[1] Two characteristics that till today are central to the concept of clostridial disease. However, it is only in 1978 that an association of *C. difficile* toxin with antibiotic-associated pseudomembranous colitis and antibiotic-associated diarrhea was first reported and that *C. difficile* was discovered as an organism responsible for 20-30% of antibiotic-associated diarrhea.[2-3] Since then, an exponentially expanding number of publications can be found describing the epidemiology, pathophysiology, risk factors, diagnosis and treatments of *C. difficile* infection (CDI).

In this introduction, we will briefly summarize some important epidemiological and pathophysiological characteristics to help frame the importance of adequate diagnosis and choice of diagnostic assay.

Epidemiology

C. difficile can be found in the gastro-intestinal tract of both humans and animals and *C. difficile* spores are ubiquitous in the environment. Spores can survive disinfectants, high temperatures and dehydration, allowing them to survive for months. Infection or colonization typically occurs via the fecal-oral route, when spores are accidentally ingested.[4]

The clinical spectrum of *C. difficile* infection (CDI) ranges from asymptomatic carriage to a potentially life-threatening pseudomembranous colitis and toxic megacolon. Carriage rates reported in literature vary significantly due to the definitions and diagnostic methods used, as well as the populations studied and the time at which the analysis was performed. Carriage rates in healthy adults have been estimated to range from 4-15%, depending on risk factors such as hospital-based employment. Asymptomatic persons are known to be a reservoir of *C. difficile*.[5-6]

Special mention should be made of infants, where asymptomatic carriage of *C. difficile* has been described in 30-70% during the first months of life, followed by a gradual decrease to adult levels by the 2-year mark. [7-9]

Risk factors associated with CDI include immunosuppression, broad-spectrum antibiotic therapy, gastric acid suppression, old age (>65j), serious underlying disease, length of hospital stay and ICU care. All antibiotic classes can elicit CDI, but clindamycin, cephalosporins and fluoroquinolones are most often described. [10]

In the early 2000's, several reports revealed disturbing increases (2x to 15x) in the incidence of CDI worldwide. Additionally, these cases involved more severe disease, higher mortality, more complications and more relapses. This phenomenon has retro-actively been explained by the spread of hypervirulent ribotypes, such as ribotype 027, which displays a high level of fluoroquinolone resistance and increased production of toxins. Other contributing factors are speculated to be an increase in the use of (certain classes of) antibiotics, a larger population at risk (>65j) and more sensitive diagnostic tests (NAAT). [11-13]

The increasing incidence as well as appearance of hypervirulent strains prompted the Scientific Institute of Public Health of Belgium (WIV) to initiate a national surveillance program in 2007. For general hospitals participation was made mandatory by the royal decree of 26/06/2007. Since this obligation was suspended 7 years later by the royal decree of 08/01/2015, participation has been dwindling (143 in 2008 => 81 in 2022) but still allows us to make use of qualitative data regarding the prevalence of CDI in Belgium. [14]

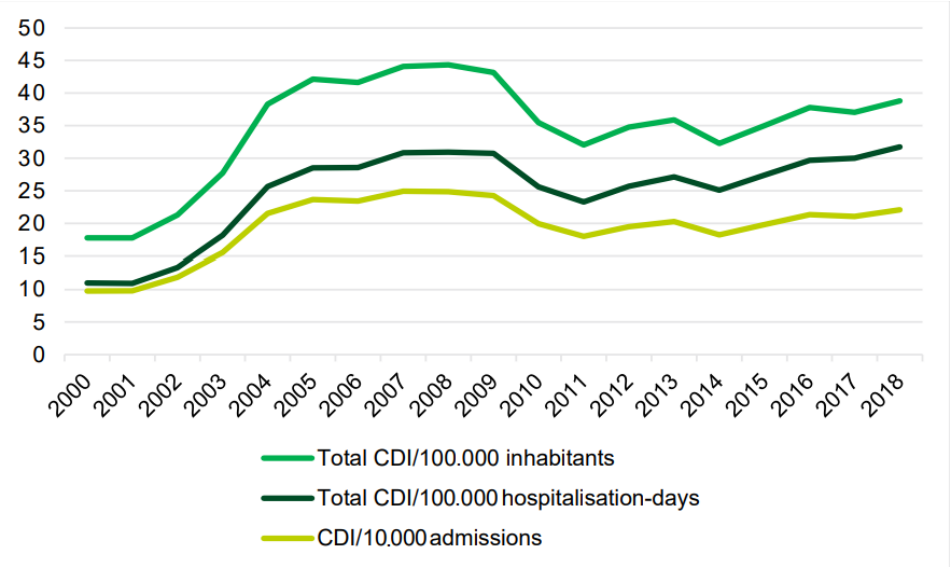

Figure 1. A summary of the incidence of CDI in Belgium from 2000-2018. Source: Federal Public Service of public health. Based on the number of ICD-9 and ICD-10 codes included in the hospital stay database. Adapted from Mortgat et al. Epidemiology of Clostridioides difficile infections in Belgian hospitals: 2020 Report. Sciensano.

In 2019 167.000 laboratory requests (86.000 hospitalized; 81.000 ambulatory) investigating the presence of toxicogenic Clostridium difficile were billed to the Belgian Government for Health Insurance (RIZIV). This amounts to 19 tests performed per CDI diagnosed in hospitals in 2019 (Positivity rate of 5.3%). [14]

Depending on whether symptoms occur within 48 hours of admittance to a hospital, a distinction is made between hospital-associated CDI (HA-CDI) and community-associated CDI (CA-CDI). In recent years an increase in CDI originating in the community has been observed in various countries (29-41%). [14-16]

In 2019 4225 cases were registered in Belgium, of which 56% were estimated to be HA-CDI. Recently a large-scale meta-analysis has estimated the CDI-related cost to healthcare at 23.329 (90% CI [12.520-34.141]) for a CA-CDI episode and 53.487 US dollar (90% CI [42.054-66.326]) for a HA-CDI episode. Applying these averages to our national situation at the current conversion rates results in an estimated annual cost of 163 million euro. [17]

Molecular typing (differentiating *C. difficile* beyond the species level) is important to recognize ongoing outbreaks and facilitate infection prevention measures. The technique most often used in Europe is capillary-based polymerase chain reaction where 16S and 23S ribosomal intergenic spacer sequences are amplified. Several ribotype classification systems exist. In America, a different typing method (pulsed-field gel electrophoresis) is frequently used, complicating comparisons. As sequencing technologies become cheaper and more available, techniques such as multilocus-sequencing and whole genome sequencing have the potential to replace existing methods due to high resolution that can be obtained. Due to the complexity of these techniques and the expertise needed to interpret results, these investigations are typically performed in a centralized reference laboratory.[18]

Because the focus of typing systems is not on providing a clinical diagnosis, these will not be discussed further in this text. Interested readers are referred to the review from Knetsch et al on the history and future perspectives of molecular typing of *C. difficile*.[18]

Pathophysiology

When the protective function of the normal microbiota is disturbed, *C. difficile* can overgrow the large intestine. The most frequent cause of bacterial dysbiosis is administration of broad-spectrum antibiotic therapy. Several enzymes and bacterial cell surface-associated proteins facilitate adhesion of bacteria to colonic epithelial cells. [4]

Symptoms correlate with the presence of a toxin-encoding pathogenicity locus (PaLoc). This PaLoc encodes 2 large exotoxins (Toxin A and Toxin B) as well as several other proteins needed for production and secretion of these toxins. Toxin A and Toxin B are some of the largest exotoxins reported to date (308kDa and 270kDa respectively). They can attach to and enter colon epithelial cells via receptor mediated endocytosis. Subsequently, they induce an inflammatory cytokine response accompanied by fluid secretion, disruption of tight junctions and cell death. [4-19]

C. difficile strains can express both Toxin A and Toxin B, B-toxin without A-toxin or no toxins at all. Nontoxicogenic strains do not cause CDI. [19]

A working hypothesis for the high asymptomatic carriage rates in infants is that infants lack the necessary receptors needed for internalization of Toxin A and B. However, more research is needed to elucidate the underlying mechanism. [7,19]

Some strains (6-12%) also produce a third toxin (CDT, binary toxin) that is encoded in the CdtLoc, a separate locus from the PaLoc. The mechanism of action and role of this toxin is still unclear, however the fact that it is increasingly found in CDI strains in patients with a severe clinical course indicates a significant role for this toxin. [19]

Current work-up in UZLeuven University Hospitals

Stool samples received for *C. difficile* analysis are first evaluated macroscopically. Liquid and semi-solid samples are homogenized and tested with the C. diff Quik Chek Complete (Techlab, USA) as per the manufacturer's instruction. Solid/formed samples are rejected, and analysis is not performed.

The C. diff Quik Chek Complete is a rapid membrane enzyme immunoassay for the simultaneous detection of *C. difficile* glutamate dehydrogenase and Toxin A and B in a single reaction well.

A negative result for both GDH and toxin A+B is interpreted as the absence of *C. difficile*. A positive result for both GDH and toxin A+B is interpreted as the presence of *C. difficile*. A discordant result for GDH and toxin A+B is arbitrated by the Xpert C. difficile (Cepheid).

The Xpert C. difficile assay is a qualitative in vitro assay for the rapid detection of toxin B and binary toxin on fecal samples of patients with a suspected CDI. It is a reverse transcription PCR (RT-PCR). A positive result is interpreted as the presence of toxigenic *C. difficile*, while a negative result is interpreted as the absence of toxigenic *C. difficile*.

There are no mechanisms in place to restrict repeat testing.

Questions

1) What assays are currently available and recommended to perform for the laboratory diagnosis of *Clostridioides difficile* Infection?

2) How has the nature and amount of requests evolved throughout the last decade in University Hospitals Leuven?
3) What are possible changes that could be made to the current workflow considering the information gathered in question 1 & 2?

Appraisal

Question 1: What assays are currently available and recommended to perform for the laboratory diagnosis of Clostridioides difficile Infection?

Diagnosing CDI is a clinical diagnosis, supported by laboratory findings. Several laboratory tests exist for assessing the presence of *C. difficile* in fecal samples. However, there is no single assay that is ideal for diagnosing CDI. Enzyme immunoassays and nucleic acid amplification tests (NAAT) are most used in routine microbiology laboratories, often making use of sequential and algorithmic testing to improve diagnostic performance. [4,20]

We can divide assays in 2 categories:

1) Assays detecting the presence of *C. difficile* such as bacterial culture, glutamate dehydrogenase (GDH) and PCR.
2) Assays detecting free toxin such as cell culture cytotoxicity neutralization assays and enzyme immunoassays

Bacterial culture followed by toxigenic assay and cell culture cytotoxicity neutralization assay (CCCNA) are both considered reference methods against which other index tests are compared. However, toxigenic culture is more often positive (owing to colonization by a potentially toxigenic strain without detectable free toxin) compared to CCCNA, which detects pre-formed toxin in faeces. A large study by Planche et al. demonstrated that CCCNA positivity, but not TC positivity correlated with clinical outcome. [21]

Toxigenic Culture (TC)

Bacterial culture with subsequent toxigenic testing of suspicious colonies is a reference method against which other methods can be compared.

Culture requires the inoculation of a fecal sample on a culture medium, followed by prolonged anaerobic incubation and the identification of suspicious colonies. Culturing times can range from 2-7 days, depending on the technique used and amount of subcultures needed. Heat or ethanol treatment of samples are ways to improve *C. difficile* isolation rates by inhibiting commensal flora and selecting spores. [22]

C. difficile will usually show good growth on non-selective agar such as commercially available anaerobe blood agar, Brucella agar with 5% blood and Columbia agar. Cycloserine-cefoxitin-fructose agar can be used as a selective medium. The presence of D-cycloserine and cefoxitin will inhibit growth of most of the Enterobacterales, anaerobic gram-negative bacilli, streptococci and staphylococci, while the ability of *C. difficile* to ferment fructose allows it to thrive. Several additives such as taurocholate and egg yolk can be added to aid in spore germination. [20,22]

More recently, chromogenic agars (E.g., ChromID C. difficile chromogenic agar (BioMérieux); CHROMagar C. difficile (CHROMagar) have been developed for the detection of *C. difficile*. Several studies have shown a superior sensitivity of chromogenic media to the comparator media.

Chromogenic media provide effective isolation within 24 hours of incubation, providing a faster alternative to the more traditional culture methods (See Appendix 1). [23-32]

A study from Hong Kong (Chen et al) emphasized the importance of confirmation of identification by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). In their study they demonstrated that 30/148 suspect colonies on ChromID CD agar could be identified as other Clostridiaceae such as *Hungatella hathewayi* (previously known as *Clostridium hathewayi* and *Clostridium clostridioforme*). [32]

Some *C. difficile* strains will not generate colored colonies due to the absence of the β-glucosidase gene. Connor et al. reported that this is a consistent feature of *C. difficile* strains belonging to ribotype 023 (UCL typing class 4). The white colonies, while still displaying typically irregular edges, can be easily missed in a routine work-up. [33]

Culture alone does not allow differentiation between toxicogenic and nontoxicogenic strains. Subsequent testing of isolates for toxin production is essential in order to diagnose CDI. [20]

A significant downside of TC is the turn-around-time (TAT). Due to the need for incubation it takes at least 24-48 hours to obtain colonies, which then still need to be tested for toxin production. This delay in diagnosis is a reason why many guidelines do not routinely recommend the use of TC. See appendix Table 2 for a summary of recommendations made by various renowned institutions. TC is mostly mentioned as an impractical gold standard, or as a potential second- or third-line assay to arbitrate differences. A notable exception is the recommendation made by the Superior Health Council of Belgium, which recommends a 2-step algorithm followed by TC for every sample to verify the results. The guideline describes several other acceptable algorithms but claims every algorithm not incorporating TC has lower sensitivity. [20, 34-40]

Glutamate dehydrogenase (GDH)

GDH is an enzyme that is strongly expressed by all strains of *C. difficile*. GDH assays are available as microwell EIA, lateral flow immunochromatographic assay, chemiluminescent immunoassay (CLIA) and enzyme linked fluorescent assay (ELFA). In 2016, a large meta-analysis conducted by Arimoto et al. evaluating 42 cohorts with 29243 comparisons showed excellent overall test performance with an overall sensitivity of 0.911 (95% CI [0.871-0.940]) and specificity of 0.912 (95% CI [0.892-0.928]). Assuming a pre-test probability of 15-25%, this would result in a positive predictive value (PPV) of 65-78% and a negative predictive value (NPV) of 97-98%. Furthermore, subgroup analysis showed similar results for the 3 most used assays (Chek-60 (Techlab), Quik Chek (Techlab) and Triage (Biosite, No longer in production)). [35,41]

Because of high sensitivity and high negative predictive value (NPV) as well as a fast TAT (15-90 minutes), many guidelines recommend the use of GDH as a potential screening test (See Appendix Table 2). Assays are typically easy to perform, with minimal hands-on time and significantly lower costs compared to NAAT (4-8€ vs 18-40€). [34-40]

Nucleic acid amplification tests (NAAT)

NAATs include assays that use polymerase chain reaction (PCR), helicase-dependent amplification (HDA) and loop-mediated isothermal amplification (LAMP). Assays typically detect the conserved regions within the genes encoding Toxin A and/or Toxin B. A recent meta-analysis performed by the European Society of Clinical Microbiology and Infectious diseases (ESCMID) included 14 cohorts and showed excellent assay performance with an overall sensitivity of 0.96 (95% CI [0.93-0.98]) and specificity of 0.94 (95% CI [0.93-0.95]). NAATs from different manufactures performed similarly. Assuming a pre-test probability of 5-10% results in a PPV of 46-64% and a NPV of 100%. [35]

The relatively lower specificity of NAAT carries a risk for overdiagnosis, especially if used as a stand-alone test in a low pre-test probability population. Overdiagnosis can have concerning consequences, such as the unnecessary use of antibacterial agents for CDI treatment. Surveillance data from Israel shows that 18.3% of 208 isolates were measured resistant to metronidazole. Another concern is the acquisition of vancomycin resistant enterococci (VRE). Oral vancomycin therapy has been shown to promote VRE overgrowth and new detection of VRE was seen in 8-31% of patients. Lastly, there are substantial financial complications whenever hospitals stays are extended for CDI treatment. [42-43]

Due to its excellent sensitivity, NAAT is often recommended as a first line screening assay alternative to GDH. While NAAT targets toxin encoding genes, positivity does not in fact prove there is in vivo production of toxin. A confirmatory assay demonstrating the presence of toxin is recommended. [20,34-40]

The Infectious Disease Society of America (IDSA) suggests that when the pre-test probability can be increased by implementing hospital wide submission criteria (Stringent exclusion of other causes and following proper indications) a single PCR could potentially be used. [34]

Cell Culture Cytotoxicity Neutralization Assays (CCCNA)

CCCNA is considered the second reference standard for diagnosing CDI against which other index tests can be calibrated. It should be noted that results can differ from the other reference test (TC). [20]

There is no single agreed upon standard method for performing a CCCNA. The core concept involves inoculating a filtrate of stool onto a monolayer of an appropriate cell line and observing for cytopathic effect after 24-48 hours after incubation at 37°C. Various kinds of different cell lines have been used in literature (Human fibroblasts, Vero cells, etc.)[20]

Once cytopathic effects have been observed, neutralization is required to prove the specificity of the cytopathic effect. This is performed using *C. difficile* antisera. Different kinds of cytopathic effects are seen due to infection with Toxin A and Toxin B combined or alone. [20]

Due to the complicated work-up, long TAT and need for cell cultures, this technique has decreased in popularity in most clinical laboratories. It is mentioned in guidelines as a gold standard but recommended only sparingly. [34-40]

Toxin enzyme immune assay (Toxin EIA)

Toxin EIA are available in many formats such as solid phase microwell, chromatographic cassettes, lateral flow membranes and automated systems using an enzyme linked fluorescent assay (ELFA) or chemiluminescent immunoassay (CLIA). A large meta-analysis investigating performance of various commercial Toxin EIAs demonstrated a pooled sensitivity of 83% (95% CI [76-88]) and 57 (95% CI [51-63]) compared to CCNA and TC respectively. Only small differences were observed between products from different manufacturers. The pooled specificity was 99% (95% CI [98-99]). The sensitivity of these assays is unacceptably low, and therefore their use as a stand-alone test is not recommended. [35]

Several articles describe more sensitive techniques such as digital ELISA's making use of single-molecule array technology to more accurately measure small amounts of toxin. Initial validation studies reported 90-100% sensitivity compared to CCCNA. An additional benefit of these assays could be the ability to quantitively determine toxin levels, if a correlation between toxin levels and clinical course could be proven. [44-46]

While more clinical studies are needed to investigate the accuracy and clinical performance of these techniques, they should prove a valuable addition to *C. difficile* diagnostic workflows in the future. [47]

Due to their specificity, toxin EIAs are recommended as a second line test by almost all society guidelines (See Appendix Table 2). [34-40]

One test vs algorithmic approach

In an ideal scenario, a single rapid test would be able to reliably predict disease status. A rapid CDI diagnosis results in fast initiation of CDI treatment and hygienic precautions, potentially avoiding complications and local spreading. However, if we were to rely solely on the most specific rapid assays such as toxin EIA (CDI prevalence of 5%, PPV of 81%), an unacceptably large group (19%) of patients with a positive result would not have CDI. This would result in a significant amount of wasted resources due to unnecessary treatments and isolation procedures.

Neither GDH nor NAAT can differentiate carriers from CDI patients. These assays cannot be used as standalone assays because an asymptomatic carrier of *C. difficile* can develop diarrhea due to a multitude of other causes. [4;20;35]

As none of these assays is suited for a standalone approach, it is best to combine tests in an algorithmic approach. The most efficient flow should attempt to minimize the total amount of assays used, while returning a result as fast as possible. This can be achieved by initially using an assay that most reliably and quickly classifies a sample as negative (high NPV – GDH/NAAT), so that no further tests are needed when a negative result has been generated. Subsequently, a fast highly specific assay (High PPV - Toxin EIA) can reliably classify samples with 2 positive results as likely CDI. There is controversy on how to best deal with discordant results (GDH positive – Toxin EIA negative). This could be the result of CDI with undetectable toxin level (possibly due to pre-analytical factors/degradation), false-negative toxin EIA results or carriage of a nontoxigenic *C. difficile* strain. Society guidelines recommend to either arbitrate with NAAT (if not used as an initial screening test), TC or a repeat sample. Some guidelines suggest to only arbitrate discordant results with a high clinical suspicion. [34-40]

Question 2: How has the nature and amount of requests evolved throughout the last decade in University Hospitals Leuven?

Amount of requests

We queried all requests for *C. difficile* analysis since 01/01/2010. Important historic changes are the implementation of GDH and Toxin testing in April of 2009 and the implementation of an arbitrating PCR in October of 2013. The current workflow (C. diff Quik Chek Complete followed by Xpert C. difficile assay) has been used continuously since 2014.

The amount of requests has more or less remained stable since 2010 and is illustrated in Figure 2. It briefly reached a minimum of 5115 in 2013 and peaked to 5951 in 2021, but no significant trends have been observed. This is in accordance with the findings of national surveillance, which indicates a consistent increase in ambulatory testing, while testing in hospitalized testing remains at the same level. [14]

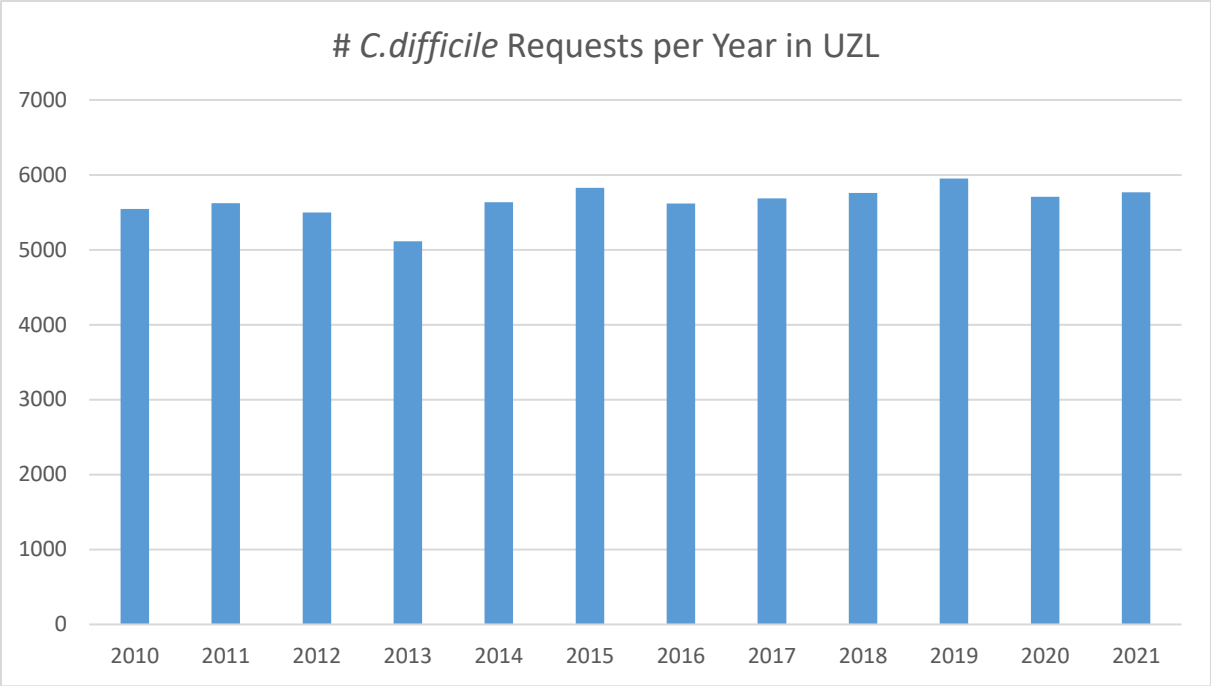

Figure 2: A summary of the amount of requests for *C. difficile* analysis in UZL from 01/01/2010 to 31/12/2021.

We analyzed how many tests were performed for each unique patient (Appendix 3). We calculated the time between successive requests of a patient to estimate how likely it was that multiple requests occurred during the same disease episode versus in different disease episodes during the year (relapses/recurrences). We found that 4%, 38% and 59% of follow-up requests occurred within 1, 7 and 14 days respectively (Appendix 4). Of these request analyses, the exact same result was obtained for 90,8%, 85,8% and 86,2% respectively (Appendix 5). The IDSA guideline explicitly recommends to not test repeat samples within a 7-day period. Other guidelines recommend to not perform repeat-testing during the same disease episode, without specification of an upper or lower limit. [34]

Positivity Rate

We analyzed the positivity rates of our diagnostic algorithm during the period 2014-2021 (See table 1) to estimate whether tests are requested with abnormal frequency compared to the national average (5.3%). The positivity rate has been decreasing the past years and now approximates the national average. It is impossible to assess whether this decrease reflects subtle changes in the requesting habits or is a result of intensified hygienic control or improved antibiotic stewardship during these years. [14]

	2014	2015	2016	2017	2018	2019	2020	2021
Total	5633	5829	5616	5685	5757	5951	5706	5769
GDH pos/Tox pos	297	202	148	180	140	210	197	195
GDH neg/Tox neg	4737	5029	4927	5023	5080	5218	5083	5198
GDH pos/Tox neg	591	581	536	474	534	505	426	372
GDH neg/Tox pos	6	16	5	8	3	18	0	4
PCR pos	276	244	270	222	275	240	186	186
PCR neg	321	361	272	260	262	285	239	190
Positivity rate	10.2%	7.7%	7.4%	7.1%	7.2%	7.6%	6.7%	6.6%

Table 1: A summary of results of the C. difficile work-up from 2014-2021 in UZL
GDH: Glutamate dehydrogenase, PCR: Polymerase chain reaction.

Workflow evaluation

An important observation that can be gleaned from table 1 is that almost all samples that tested negative for GDH also tested negative for toxins. Furthermore, of the arbitration PCRs performed on GDH neg/ Tox pos samples only 6/60 (10%) tested positive. Medical chart review of those 6 cases revealed 3 CDI cases and 3 cases without clinical symptoms suggestive of CDI. We can conclude that performing GDH first, and only testing toxins if GDH testing is positive would have missed only 3 cases out of 45946 (0.0065%) requests.

Separating GDH and toxin analysis results in increased hands-on time when laboratory workers are prompted to perform a second test of any kind. Especially when considering relatively cheap assays, the proportion of total cost resulting from manual actions (E.g., finding samples, pipetting, reading and entering results) is significant. Assuming an hourly cost of 49,2 euro, 4 minutes of labor costs 3,28 euro. A significant decrease in reagent cost is necessary to compensate for the extra time spent sorting the samples needed and performing the additional tests.

Automated sample analyzers such as the Liaison® analyzer (DiaSorin) can perform reflex testing automatically, without the need for extra hands-on-time. However, this advantage comes with a trade-off. There is a cost associated with the acquisition and maintenance of the analyzer itself, as well as with the purchase and running of calibration materials. The frequency of calibration is another issue, as delaying tests in order to perform larger batches has a profound impact on the clinical management. [48-49]

The detection of toxigenic *C. difficile* in faeces is reimbursed by RIZIV via code 549850 / 549861. It has a B-value of 800 and can only be charged to patients older than 2 years (conversion rate 0,032504 since 01/01/2022; 26 euro). [50]

Requests in children younger than 2 years

	2014	2015	2016	2017	2018	2019	2020	2021
Total	5633	5829	5616	5685	5757	5951	5706	5769
Amount 0-2y	553	374	331	334	497	361	257	234
%	9.8%	6.4%	5.9%	5.9%	8.6%	6.1%	4.5%	4.1%

Table 2: Summary of the amount of *C. difficile* requests in children younger than 2years in UZL.

We have calculated the amount of tests that do not qualify for RIZIV reimbursement for each year (Table 2). Guidelines state there is little evidence to perform *C. difficile* testing on diarrheal samples in infants <2 years. Requests should only occur after other causes have been sufficiently excluded, and in the presence of risk factors. The amount of requests in this population has seen a clear decrease in the past years, however, 4.1% remains a rather significant portion of tests. Positivity rates in these samples are summarized in Appendix 7 and are significantly higher compared to all samples (25.7 to 11.5%, decreasing trend). However, the clinical relevance of these results is unclear. As a tertiary center, we provide care for a substantial number of immunocompromised infants, this might partly explain the number of requests in young children.

Macroscopic evaluation

Year	2014	2015	2016	2017	2018	2019	2020	2021
Total	5633	5829	5616	5685	5757	5951	5706	5769
No description	1060	842	863	1003	933	2486	3975	4008
Semi-solid	2801	3035	3041	2933	3189	2196	1029	1011
Liquid	1274	1402	1341	1376	1287	1051	561	566
Slimy	313	385	254	279	318	164	93	86
Formed	333	313	223	238	158	151	99	137
Bloody	73	87	71	63	70	41	24	33

Table 3: Summary of macroscopic evaluations reported for *C. difficile* requests in UZL. Some samples were described using more than one descriptor.

The macroscopic evaluations registered in the laboratory information system for all requests have been summarized in table 3. The past years there has been an enormous increase in the amount of samples for which no macroscopic evaluation has been registered (2021: 4008/5769; 69.5%), this complicates any analysis investigating differences. Additionally, a significant albeit decreasing, number of tests was performed on formed stool samples, which has no clinical relevance. [4,34-40]

While observing laboratory workers, it became clear that it was not possible to register the macroscopic evaluation of a stool specimen if that sample had only been sent for *C. difficile* analysis. This could explain the sudden surge of samples without a description.

No significant differences in positivity rates were found between the macroscopic evaluations (Data not shown).

Conclusions

The current working procedure of UZL is in accordance with almost all international guidelines. It is supposedly a less sensitive algorithm, according to the guidance of the Belgian Superior Health Counsel, due to the absence of toxigenic culture. The ESCMID guidelines also states that every laboratory should be able to isolate *C. difficile*, something that is not possible at this moment. [35]

It should be noted that due to a decrease in participation of the national surveillance and a shift towards NAAT the Belgian reference center for *C. difficile* accepts the submission of stool samples as well as pure cultures. This reduces the burden of peripheral laboratories to maintain culture methods for *C. difficile*.[51]

Optimalization of the testing algorithm is possible. This data should be considered carefully when evaluating alternative tests.

To Do

Contact IT to enable laboratory workers to register the macroscopic evaluation of stool samples for *C. difficile* analysis and to provide specific annulation for solid stools.

Remind laboratory workers to reject formed/solid stool samples.

Evaluate the cost-effectiveness of commercially available GDH assays, toxin assays and PCR assays for the current situation of UZL.

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Appendix 1. Summary of studies comparing ChromID C. difficile with other culture media for isolation of *C. difficile* from stool samples. Adapted from Perry et al.

Author/ reference	Year	#samples/ positive samples	Sample treatment	Test media	Sensitivity (%) evaluated at	
					24u	48u
Eckert et al. / 29	2013	406/54	None	ChromID	74.1	87
				TCCA		85.2
				CLO		70.4
Carson et al. / 26	2013	50/47	None	ChromID	100	
				TCCFA		87
		100/96	Alcohol	ChromID	99	
				TCCFA		96
Yang et al. / 31	2014	289/49	None	ChromID	93.9	98
				CCFA	18.4	30.6
Han et al. / 27	2014	185/36	Heat	ChromID	58.3	100
				CDSA		83.3
Shin et al. / 28	2014	530/180	Alcohol	ChromID	55.6	85
				CDSA	19.4	75.6

TCCA, brain heart infusion agar plus 5% blood, taurocholate, cycloserine, and cefoxitin; CLO, Clostridium difficile agar (bioMérieux); TCCFA, cycloserine-cefoxitin-fructose-egg yolk agar (CCFA) plus 0.1% taurocholate; CDSA, C. difficile selective agar (BBL); CCFA, cycloserine-cefoxitin-fructose-egg yolk agar

Appendix 2. Summary of recommendations of diagnostic guidelines for *C. difficile*.

Society	Guideline	Country	Year	Target population	Recommended testing strategy	Repeat testing	Infant	Culture
Infectious diseases Society if America (IDSA)/ Society for Healthcare Epidemiology of America (SHEA)	Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children	America	2017	Unexplained new-onset ≥3 stools in 24hours	Stool toxin test in multistep algoritm (GDH + toxin; GDH + toxin with NAAT arbitration; NAAT + toxin)	Do not perform repeat testing withing 7 days and do not test asymptomatic patients	Do not routinely test infants ≤12 months of age with diarrhea. (1-2y only after exclusion of other causes)	Gold standard, not recommended for routine work-up
American College of Gastro-enterology (ACG)	Clinical Guidelines: Prevention, Diagnosis, and Treatment of Clostridioides difficile Infections	America	2021	3 or more unformed stools in 24 hours	CDI testing algoritms should include a highly sensitive (GDH/NAAT) followed by a highly specific testing modality. (Toxin EIA) Arbitration reserved for patients with high clinical suspicion.	No mention	No mention	Gold standard, but impractical outside of research setting
Australian Infection Control Association (AICA)	Infection control guidelines for patients with Clostridium difficile infection in healthcare settings	Australia	2011	Only perfomed on unformed stools unless ileus is suspected.	Combination of a sensitive and specific test.	Not recommended during the same episode.	No mention	No mention
European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for C. difficile (ESGCD)	European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for Clostridium difficile infection	Europe	2016	All unformed fecal samples and rectal swabs in case of ileus.	2 stage algorithm consisting of a screening test with high sensitivity (NAAT or GDH) followed by a highly specific (Toxin EIA) test. NAAT or TC as arbitration of discordant results based on clinical evaluation or local infection prevention guidance.	Not routinely recommended during the same episode, justifiable during outbreak (lower NPV due to higher prevalence). Test of cure is not recommended.	Do not routinely test infants ≤3 years. Testing reserved for physician's request.	Every laboratory should be able to isolate C. difficile
Hoge gezondheidsraad (HGR)	Recommendations for the control and presvention of *Clostridium difficile* in health care facilities.	Belgium	2019	Fresh and liquid stool. Swabs have insufficient volume to perfom testing.	3-step algorithm consisting of GDH+Toxin IA arbitrated by PCR (prelimenary result) followed by TC the next day.	No mention	No mention	Present in the recommended approach
Scottish Health protection Network	Guidance on Prevention and Control of Clostridium difficile Infection (CDI) in health and social care settings in Scotland.	Scotland	2017	Restricted to diarrheal samples.	2-step algorithm consisting of GDH or NAAT followed by Toxin IA or CCCNA	Not recommended. Test of clearance is not recommended.	Do not routinely test infants ≤3 years. Testing reserved for physician's request.	No mention
RijksInstituut voor Volksgezondheid en milieu (RIVM)	Guidance document: Clostridium difficile	Netherlands	2014	restricted to diarrheal samples. Swabs are inadequate.	2 step algorithm consisting of GDH as a screening test followed by a test with high specificity. Arbitrated by a repeat sample, PCR or TC.	Possible to arbitrate discordant screening resuslts.	Do not routinely test infants ≤2 years.	Possible as arbitrage, not required.

Appendix 3: Summary of the distribution of *C. difficile* requests from 2014-2021

total requests	Unique patients	Year	# Requests/ Patiënt																																				
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	11	12	13	14	15	16	17	18	19	20	22	23	24	30
5633	3285	2014	2204	567	228	98	51	39	27	13	10	6	5	4	2	3	0	4	2	1	0	0	5	4	2	3	0	4	2	1	0	0	0	0	0	0
5829	3516	2015	2399	610	225	104	59	51	23	13	6	8	3	1	2	2	0	0	0	0	0	1	3	1	2	2	0	0	0	0	0	1	0	0	0	0
5616	3483	2016	2413	598	221	102	57	35	14	13	6	3	1	3	3	1	0	2	0	0	0	0	1	3	3	1	0	2	0	0	0	0	0	1	0	0
5685	3504	2017	2435	595	224	100	49	34	29	12	5	8	0	2	0	2	0	0	0	0	1	1	0	2	0	2	0	0	0	0	1	1	0	0	1	0
5757	3550	2018	2468	607	216	99	61	30	19	10	9	6	1	3	2	3	1	2	0	0	0	0	1	3	2	3	1	2	0	0	0	0	0	0	0	1
5951	3599	2019	2503	581	211	115	73	30	21	17	8	5	6	1	4	1	3	2	0	0	0	0	6	1	4	1	3	2	0	0	0	0	1	0	0	0
5706	3468	2020	2374	598	225	108	55	30	23	12	6	7	4	4	2	3	0	1	1	0	0	0	4	4	2	3	0	1	1	0	0	0	0	0	0	0
5769	3598	2021	2512	586	246	115	53	28	13	15	4	8	3	2	0	0	1	0	1	1	0	1	3	2	0	0	1	0	1	1	0	1	0	0	0	0

Appendix 4: Summary of the average time between successive requests for the same patient

	2014	2015	2016	2017	2018	2019	2020	2021
>1 day	94%	95%	96%	96%	97%	97%	97%	97%
<1 day	5%	4%	4%	4%	3%	3%	3%	3%
>7 days	55%	60%	63%	63%	63%	62%	64%	62%
<7 days	45%	40%	37%	37%	37%	37%	36%	38%
>14 days	37%	42%	42%	43%	42%	43%	41%	41%
<14 days	63%	58%	58%	57%	58%	57%	59%	59%

Appendix 5: Summary of agreement of repeat-requests (2021)

	Delta <1d	N/N	P/P	P/N/N	P/N/P
Resultaat 1	N/N	55	0	0	1
	P/P	2	1	0	0
	P/N/N	0	1	2	1
	P/N/P	1	0	0	1

	delta <7d	N/N	P/P	P/N/N	P/N/P
Resultaat 1	N/N	661	21	8	16
	P/P	15	11	2	8
	P/N/N	10	2	19	2
	P/N/P	24	5	3	11

	delta <14d	N/N	P/P	P/N/N	P/N/P
Resultaat 1	N/N	1043	31	18	19
	P/P	23	12	3	13
	P/N/N	20	3	29	2
	P/N/P	36	5	5	16

Appendix 6: Positivity rate in samples from patients aged 0-2

	2014	2015	2016	2017	2018	2019	2020	2021
Total 0-2y	553	374	331	334	497	361	257	234
pos/pos	87	17	14	18	27	25	16	11
neg/neg	356	262	259	243	363	254	197	189
pos/neg	110	95	58	73	106	82	44	34
neg/pos	0	0	0	0	1	0	0	0
PCR pos	55	34	28	26	58	28	17	16
PCR neg	55	61	31	47	49	54	27	18
Positivity rate	25.7%	13.6%	12.7%	13.2%	17.1%	14.7%	12.8%	11.5%