Clostridiodes difficile infection (*Clostridioides difficile*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *clostridiodes difficile*.

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1 PHLN Summary Laboratory Definition

1.1 Condition:

Clostridioides difficile infection (CDI).

A diagnosis of CDI implies:

a. laboratory detection of *C. difficile* toxins and/or toxigenic *C. difficile* in faeces, rectal swab or bowel contents PLUS
b. relevant clinical manifestations: diarrhoea (usually defined as 3 or more loose stools in a 24 hour period) or, less commonly, ileus, toxic megacolon or pseudomembranous colitis (identified by endoscopy).

1.1.1 Definitive laboratory criteria

Direct identification of preformed *C. difficile* toxin(s) in an unformed (diarrhoeal) stool sample.

1.1.2 Suggestive laboratory criteria

a. Direct detection of gene(s) within the Pathogenicity Locus (PaLoc) (which encodes *C. difficile* toxin production - *tcdA* and/or *tcdB, tcdE)* - in an unformed (diarrhoeal) stool sample or in bowel tissue by nucleic acid amplification tests OR
b. Isolation, from an unformed (diarrhoeal) stool sample or bowel tissue, of *C. difficile*, which EITHER

- possesses one or more *C. difficile* toxin-related gene(s) carried on the pathogenicity locus (PaLoc) plus or minus the *C. difficile* transferase locus (CdtLoc), OR
- produces toxin A and/or B in vitro.

2 Introduction

2.1 The organism and its toxins

C. difficile is a Gram positive spore-forming bacterium, which was shown to be the cause of pseudomembranous colitis – a condition often associated with use of the (then) new antibiotic, clindamycin - in 1978[1]. In 2016, *Clostridium difficile* was reclassified as *Clostridioides difficile*. It is widely distributed in the environment and faecal flora of humans and animals. With rare exceptions, colonisation and infection are limited to the gastrointestinal tract.

Local and systemic symptoms are due to the effects of one or both of two toxins, encoded by genes carried on a pathogenicity locus (PaLoc), which is absent from non-toxigenic strains: toxin A (TcdA or "enterotoxin", encoded by *tcdA*) and toxin B (TcdB or "cytotoxin", encoded by *tcdB*). Although both are cytotoxic, TcdB is 100-1000-fold more potent; TcdA causes fluid accumulation, similar to cholera toxin, in the rabbit ileal loop[2]. The PaLoc also carries *tcdC* and *tcdR*, which are, respectively, negative and positive regulators of toxin production, and *tcdE*, which is involved in toxin release[3]. A minority (3-12%) of *C. difficile* strains infecting humans produce binary toxin or *C. difficile* transferase (CDT), which is a classic two component toxin with binding (CDTb) and enzyme/toxic (CDTa) subunits encoded by *cdtA* and *cdtB*, respectively; they are carried on the CDT locus (CdtLoc) along with a regulatory gene, *cdtR*. CDT production is generally associated with increased virulence of *C. difficile*, although the mechanism is uncertain; it is cytotoxic in vitro and believed to enhance adherence of *C. difficile* to the intestinal epithelium[3].

Various combinations of toxin genes occur in different *C. difficile* strains; the majority produce both TcdA and TcdB or TcdB alone, but not binary toxin. By contrast, ribotype (RT) 027 (refer below) and related strains, which originate from animals, produce TcdA, TcdB and binary toxin[2, 4].

2.2 Infection and colonisation

Symptoms of CDI range in severity from a few loose stools to severe bloody diarrhoea with colonic ulceration, pseudomembrane formation, fever, leukocytosis and systemic toxicity, sometimes further complicated by life-threatening toxic megacolon or perforation and peritonitis, requiring emergency colectomy. The average attributable mortality is reported to be ~5%, but it varies widely according to patient characteristics – particularly age – and the *C. difficile* strain involved. Recurrent CDI – i.e. diarrhoea recurring after improvement of symptoms, within 8 weeks of the onset of the original incident - occurs in around 20% of cases, on average, but this also varies. Recurrence can be due to relapse of the original infection or reinfection with a different strain, particularly in individuals with ongoing risk factors e.g. immunosuppression or a continuing or repeated antibiotic therapy[1].

Asymptomatic carriage of *C. difficile* (including toxigenic strains) is not uncommon, particularly in young children or, in adults, after a period of hospital admission and among residents of long term care facilities; carriage often persists for several weeks after symptomatic recovery from an episode of CDI[6]. Up to 50-60% of healthy infants are colonised with *C. difficile* in the first year of life; this is most

likely if establishment of the normal gut microbiome is delayed or modified because of Caesarean section delivery, exposure to antibiotics or artificial feeding. Infants are resistant to the effect of *C. difficile* toxins, apparently because they lack relevant epithelial receptors, and rarely develop symptomatic CDI[7].

Colonisation rates decline in the second year of life as the gut microbiome becomes more complex and immune competence matures and is uncommon in healthy older children and adults (3-5%). Subsequent exposure can result in asymptomatic carriage or CDI, in individuals who are susceptible because of waning immunity - due to increasing age or immunosuppression associated with chronic illness or chemotherapy – and/or disruption of gut microbiota due to antibiotic therapy - especially with broad spectrum agents, such as clindamycin, third or fourth generation cephalosporins or fluorquinolones. Gastrointestinal surgery, inflammatory bowel disease, acid suppressive therapy, especially with protein pump inhibitors, and excessive use of laxatives or enemas are all associated with increased risk of CDI in some studies[6, 8].

2.3 Transmission

C. difficile is spread via the faecal-oral route by ingestion of spores, which are resistant to drying, heat and many disinfectants and so persist in the environment, particularly in the vicinity of individuals with diarrhoea due to *C. difficile*. CDI was once thought to be almost exclusively healthcare-associated, with spores spreading, via a contaminated environment or on the hands of healthcare workers, from a patient with diarrhoea to other susceptible patients, who would subsequently develop similar symptoms. It is now recognised that at least one third of cases are community-acquired[9, 10] and the organism can spread from asymptomatic carriers, including colonised infants who may be an important reservoir of community-acquired infection[10]. The strains they carry are often toxigenic and similar to those that cause CDI in susceptible adults[07]. Whole genome sequencing has demonstrated that *C. difficile* strains causing CDI – even when apparently hospital-acquired (i.e. those with onset of symptoms >48 hours after admission) - often cannot be genetically linked to another symptomatic patient or other hospital or community source, indicating that sources are diverse and often unrecognised[11]. Some apparently hospital-acquired infections presumably result from an existing carrier being exposed, after admission to hospital to risk factors such as antibiotic therapy or bowel surgery.

C. difficile can colonise and cause diarrhoea in food-producing, especially young, animals; meat and poultry at the point of sale are not infrequently contaminated with *C. difficile*; vegetables can be contaminated by animal manure and ready-to eat food can be contaminated during preparation. Even low-level contamination with spores may resist short periods of cooking. Food-borne transmission is probably a significant source of community-acquired CDI, but is difficult to prove in individual cases[12, 13]. The distribution of *C. difficile* strains (RTs) that colonise and infect animals differs from that of those that are most prevalent in humans, but there is overlap and direct transmission between animals and humans e.g. RT 078 has been postulated[14].

2.4 Pathogenesis

After ingestion, spores survive the acid environment of the stomach, germinate on exposure to bile in the small intestine and proliferate in, and colonise or infect, the colon[4]. In favourable circumstances – disruption of the gut microbiota and/or reduced immunity - *C. difficile* penetrates the mucus layer and

adheres to the colonic epithelium. Toxin production increases during the stationary growth phase; cellular intoxication causes disruption of the cytoskeleton and tight junctions, increased epithelial permeability, fluid accumulation and an intense local and systemic inflammatory response. Toxin B (and, to a lesser extent, toxin A) alone can produce these effects but toxins A and B usually act together. Disease severity varies with different *C. difficile* strains; binary toxin production and differences in germination, sporulation and biofilm production between strains can contribute to virulence[4].

2.5 Evolving CDI epidemiology

For some time CDI was regarded as a relatively uncommon cause of diarrhoea, occurring mainly in elderly hospitalised patients with multiple co-morbidities. Outbreaks were reported, but uncommon. In 2004, a report from Quebec[15], drew attention to a dramatic increase in the incidence, severity and mortality of CDI in the region, including in younger (<65 years) age-groups. Similar increases had occurred in some parts of the USA[16] and, in both countries, were associated with emergence of a previously rare, fluoroquinolone-resistant *C. difficile* strain[16, 17], identified by pulse-filed gel electrophoresis (PFGE) as NAP1, restriction endonuclease analysis (REA) as BI, ribotyping as RT 027 and toxinotyping as toxinotype III.

This strain (now generally referred as 027) is characterised by production of binary toxin/CDT, and an 18-bp deletion at position 117 (tcdCD117) in *tcdC*, which affect the negative regulatory function of TcdC and lead to increased toxin production[6]. Most, but not all, studies show that CDI due to RT 027 and other strains with similar toxin profiles (mostly belonging to clades 2 and 5) are associated with higher complication rates and mortality than strains with more typical profiles[18].

C. difficile RT 027 rapidly spread to the UK where widespread hospital outbreaks occurred, and subsequently to several European countries[19]. In most countries, in which major hospital CDI outbreaks due to RT 027 have been documented, it has become the predominant strain. However, after the incidence and mortality from CDI in the UK reached a peak in 2006-8, major national infection control programs were implemented throughout the UK, which resulted in sharp falls in CDI rates.

C. difficile RT 027 was also associated with an increase in the incidence and recognition of community-acquired infections, not only due to RT 027, but also less virulent strains. In some European countries, another virulent RT, 078, which is commonly associated with animals, especially pigs, has been increasingly recognised causing community-acquired infection in humans[14]. Typically, community-acquired infections occur in younger age-groups than hospital-acquired CDI, often in the absence of obvious risk factors; because those affected generally have fewer comorbidities, the overall mortality is lower than that of hospital-acquired infection, but up to 40% of patients with community-acquired CDI require admission to hospital and rapidly progressive, severe disease can occur in previously healthy young people[5, 10].

There has been relatively little spread of RT 027 in Australia; only a few individual imported[20] and hospital-acquired[21] cases have been reported. However, limited strain typing of *C. difficile* isolates has identified the emergence of several previously unknown RTs with increased virulence with toxin profiles similar to and belonging to the same clade as RT 027, such as RT 244[22] and RT 251. In addition, a multitude of clade 5 strains has been detected in Australian food animals but not RT 078. Prospective national surveillance of public hospital-diagnosed cases of CDI, indicate that the

incidence of CDI, including community-acquired disease, is increasing[23]. However, changes in testing criteria and laboratory methods have probably contributed to some but not all of this apparent increase.

2.6 Diagnosis

Indications for and methods of laboratory diagnosis of CDI have changed significantly. Previously, it was based on detection of preformed *C. difficile* toxin(s) in, or culture of toxigenic *C. difficile* from faeces; both methods are slow, relatively difficult and usually done only in response to a specific request on specimens from hospital patients. Subsequently, immunoassays for detection of toxins A and/or B and glutamate dehydrogenase (GDH) – a *C. difficile* "common antigen" - and nucleic acid amplification tests (NAATs) became commercially available and widely used. However, there is wide variation in their analytical performance and costs[2, 24, 25] and in their use in different Australian laboratories[26], which affect the reliability of diagnosis and national CDI surveillance data. A standardised approach to diagnostic testing for *C. difficile* can improve the validity of surveillance data and is recommended[27, 28].

a. **Tests for preformed *C. difficile* toxin in faeces:** The cellular cytotoxicity neutralisation assay (CCNA) was once regarded as the "gold standard" for diagnosis of CDI; however, although highly specific, its sensitivity is only 75-85% that of toxigenic culture[29]. CCNA requires cell culture capability, the turnaround time is 24-48 hours and it is now rarely performed. Preformed *C. difficile* toxins A and/or B can also be identified in stool by immunoassays. The best of the commercial assays are also specific (>95%) but less sensitive than CCNA[24]. Early assays only tested for toxin A – because a) it was thought that all toxigenic strains produced it and b) toxin A is more immunogenic than toxin B - are now recognised as unsuitable since a significant minority of *C. difficile* strains in Australia produce only toxin B (e.g. the major ribotype found in Asia, RT 017, is toxin A negative); most assays are now designed to detect both toxins A and B.
b. **Tests for toxigenic *C. difficile* in faeces:** Toxigenic culture is an alternative "gold standard" for diagnosis of CDI, which is more sensitive than CCNA, but less specific for CDI, since it detects the genetic potential for toxin production, but not necessarily actual toxin production in vivo. The turnaround time for culture is 24-48 hours and a confirmatory test for toxigenicity is required. While culture is impractical for routine diagnosis, it is required for strain typing and antibiotic susceptibility testing. Toxigenic *C. difficile* can also be detected in faecal specimens by rapid methods, such as a combination of GDH immunoassay plus supplementary immunoassay to detect preformed toxin or by direct NAAT to detect toxin genes.

The presence of toxigenic *C. difficile* is a necessary, but not sufficient, condition for diagnosis of CDI – diarrhoea in a colonised patient can be due to another cause. Although detection of preformed toxin results in fewer positive tests, it may correlate better with clinical findings. A recent prospective, multicentre study evaluated toxigenic culture, CCNA and rapid methods (alone and in combination) based on clinical measures of CDI severity including routine blood tests, length of hospital stay and 30-day mortality[30]. Results in >6,500 faecal specimens were grouped according to test results: group 1, CCNA (and toxigenic culture) positive (7% of specimens); group 2, toxigenic culture positive, CCNA negative (3%); group 3 both CCNA and toxigenic culture negative (90%). Mortality was significantly higher in group 1 (16.6%) than in either group 2 (9.7%; p=0.002) or 3 (8.6%; p=<0.0001) and not

significantly different between groups 2 and 3 (p=0.53). The authors concluded that patients from whom group 2 isolates originated from were shedding *C. difficile,* who probably did not have CDI but were potential sources of cross-infection. None of the rapid tests, in isolation, gave acceptable predictive values but the combinations that correlated best with gold standard methods were:

- Toxigenic culture: GDH EIA (Techlab C diff Chek-60 glutamate dehydrogenase enzyme immunoassay) plus PCR (GeneXpert, Cepheid): sensitivity, 92%; specificity, 98%; PPV, 81%; NPV 99%;
- CCNA: Toxin A/B EIA (Techlab C difficile Tox A/B II toxin enzyme immunoassay) plus PCR or GDH EIA plus toxin A/B EIA: sensitivity, 82%; specificity, 99.5%; PPV 92%; NPV 99%.

It is now generally recommended that the diagnosis of CDI be based on a combination of rapid tests performed sequentially or in parallel[2, 8, 28, 29, 31] and supplemented by culture in at least some larger laboratories so that strain typing can be performed for surveillance. Multistep (usually two-step) algorithms for testing (i.e. GDH plus toxin, arbitrated by NAAT, or NAAT plus toxin) rather than a toxin test alone have been advocated as a preferred approach. The choice will depend not only on predictive values and clinical validation, but also on cost, turnaround times and laboratory workflow.

2.7. Strain typing and surveillance

PCR ribotyping is the commonest method used for *C. difficile* strain typing in a small number of Australian laboratories. Other methods include toxinotyping - a restriction fragment length polymorphism assay for variations in the PaLoc of toxigenic *C. difficile* strains; multilocus sequence typing (MLST); pulse field gel electrophoresis and restriction endonuclease analysis[25]. Whole genome sequencing (WGS) has emerged as a method of typing and characterization of *C. difficile*, and is used predominantly to identify outbreaks. WGS is amenable to standardization and has radically improved the resolution of *C. difficile* typing, enabling examination of molecular epidemiology and toxigenic gene content and tracing pathogen transmission events[32]. In addition, genomic similarity (≤2 allelic differences documented by core genome MLST) between isolates recovered from the same patient with recurrent CDI suggests a relapse of infection, while difference of over 50 alleles between the initial strain and subsequent strain of *C. difficile* may indicate exogenous reinfection[33].

3 Laboratory Diagnosis/Tests

3.1. Testing criteria

- Tests for toxigenic *C. difficile* should only be performed on unformed stool specimens (or gut contents from patients with diarrhoea), unless ileus is suspected.
- All adults and children over 2 years, who have been hospitalized for >48 hours and develop diarrhoea (>3 unformed stools on a 24-hour period) should be tested for CDI.
- All adults and children over 2 years, in whom diarrhoea has persisted for >48 hours and no other enteropathogen has been identified should be tested for CDI.
- Repeat testing of faecal specimens during the same episode of diarrhoea is not recommended a) within 4 weeks of a positive test (response to treatment is determined by clinical criteria) or

b) following a negative test – unless CDI is strongly suspected and a more sensitive method (e.g. NAAT) is used after a negative immunoassay.

- Tests for *C. difficile* in children <2 years old should be performed in consultation with a paediatrician.

3.2 Cellular cytotoxicity neutralisation assay (CCNA)[27]

Cell monolayers (usually Vero or Hep2 cells) are cultured in the presence of a faecal (or culture) filtrate, with and without neutralising antitoxin antibodies (usually *C. sordellii antiserum)* and examined at 24 and 48 hours for cytopathic effect (cell rounding) that is absent in monolayers with neutralising antibody. This assay is now rarely used either for primary diagnosis or confirmation of in vitro toxin production by *C. difficile* isolates.

3.2.1. Suitable specimen

Unformed stool or culture filtrate. Specimens should be tested as soon as possible after receipt or stored at 4oC for no more than 48 hours. Prolonged storage and repeated freezing and thawing will result in degradation of toxin.

3.2.2 Test sensitivity

Compared with toxigenic culture 75-85%[29]

3.2.3. Test specificity

Compared with toxigenic culture, theoretically, 100%. However, note that neither method is standardised, both require considerable expertise and results vary between laboratories. Rarely, CCNA is positive in culture-negative specimens, which is most likely to indicate insensitive culture methods[29].

3.2.4. Predictive values

In the presence of relevant clinical symptoms, a positive result (i.e. neutralisable cytopathic effect) is generally regarded as definitive evidence of CDI, but a negative result does not exclude it. Reduced sensitivity can be due to degradation of toxin in the specimen due to delayed testing or repeated freeze-thawing.

3.2.5. Suitable acceptance criteria.

Cytopathic effect (cell rounding) produced by faecal filtrate in a cell monolayer without antitoxin but not in a monolayer with added antitoxin. Note that a very high concentration of toxin in faecal filtrate can overwhelm the neutralising capacity of antitoxin. If typical cytopathic effect is produced, but not inhibited by antitoxin, the assay should be repeated with dilutions of faecal filtrate.

3.3. *C. difficile* culture

3.3.1 Suitable specimens

Unformed faeces; rectal swab (e.g., from patient with ileus or toxic megacolon) or intestinal contents. Heat or alcohol pretreatment of the specimen is generally recommended to kill vegetative organisms and minimise overgrowth of other faecal flora, whilst sparing *C. difficile* spores.

Alcohol shock pretreatment. Equal parts (1mL) of specimen and industrial methylated spirit or absolute alcohol are homgenised in a vortex mixer and then left at room temperature of 1 hour before inoculation[35].

Heat shock pretreatment. The specimen is heated at 80oC for 10 minutes before inoculation.

3.3.2 Media

A variety of media can be used for broth enrichment and/or direct plating. Recent comparisons of media have produced contradictory results[2, 35, 37]. Those most commonly used and generally satisfactory include cefoxitin cycloserine fructose agar (CCFA), cefoxitin cycloserine egg yolk agar (CCEY), and chromogenic *C. difficile* agar. Taurocholate or lysozyme is generally added (or included in commercial media) to enhance spore germination; broth enrichment (e.g using CCFB) may[35] or may not[37] increase culture sensitivity. Most media except chromogenic *C. difficile* agar need to be prereduced in anaerobic conditions for 2 hours before inoculation. Media are incubated anaerobically at 35oC. Growth of characteristic colonies is often visible after 24 hours incubation on chromogenic agar, but recovery can be increased by incubation for up to 72 hours.

Recent comparison of prereduced taurocholate-containing CCFA (TCCFA) – as recommended by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA)[34] – with chromogenic *C. difficile* agar (CDIF, BioMerieux)[36, 38] showed that the latter was more selective (less faecal flora grown), even without alcohol shock specimen pretreatment, and more sensitive (more specimens positive and more *C. difficile* colonies on CDIF at 24 hours than on TCCFA at 48 hours), even without prereduction of plates. Several chromogenic media are available; they are more expensive but more sensitive and convenient to use than conventional media (more *C. difficile* colonies on CDIF at 24 hours than on TCCFA at 48 hours, even without prereduction of plates).

3.3.3 Test sensitivity

As indicated by comparative studies, there is considerable variability in sensitivity depending on specimen treatment, broth enrichment, media used and duration of incubation.

3.3.4 Test specificity

Isolation of *C. difficile* from faeces indicates at least colonisation; confirmation of toxigenic potential by CCNA, immunoassay or NAAT is required for presumptive or definitive diagnosis of CDI.

3.3.5 Predictive values

Negative culture does not exclude CDI (although if appropriate methods are used it makes the diagnosis unlikely). Most commonly, culture is performed only on specimens in which one or more rapid tests for *C. difficile* is positive (refer below).

3.3.6 Suitable acceptance criteria

Isolation of colonies with typical morphology (irregular, flat, with 'ground glass' appearance on TCCFA) allows presumptive identification of *C. difficile*. Black colonies typically isolated on CDIF are easily recognized, but not produced by a small minority of *C. difficile* strains (notably RT 023). Isolates should be subcultured for purity on horse blood agar, on which they produce chartreuse colony fluorescence under UV light. Further confirmatory identification is generally by nucleic acid testing and/or immunoassay for toxins A and B.

3.3.7 Suitable external QC program

No QC program is available for *C. difficile* culture *per se*, which is performed by a minority of laboratories.

3.4. Immunoassays for *C. difficile* common antigen - glutamate dehydrogenase (GDH)

GDH is a metabolic enzyme, encoded by gluD; it is produced by toxigenic and nontoxigenic strains of *C. difficile*, and cross-reacts with GDH of *C. sordellii*. A number of enzyme immunoassays (EIAs) and immunochromatographic tests (ICTs) are commercially available, with similar performance characteristics[2]. GDH assays are rapid, sensitive and inexpensive screening tests for CDI, but must be combined with or supplemented by more specific confirmatory tests.

3.4.1 Suitable specimens

Unformed faecal specimen.

3.4.2 Test sensitivity

There is considerable variation in the reported sensitivity (88-100%), which probably reflects differences in methodology between studies, including duration and methods of specimen storage before evaluation and the reference method used[2]. They are generally highly sensitive (>95%) for detection of *C. difficile*.

3.4.3. Test specificity

Specificity is also variable (76-98%), depending on methods and reference standards[2].

3.4.4. Predictive values.

GDH has a very high NPV (98-99%) but relatively poor PPV (61-94%) for CDI[2, 28]. It is generally recommended that a negative result can be reported as excluding CDI. Specimens with positive results require additional testing.

3.4.5 Suitable acceptance criteria.

Tests should be performed and interpreted according to manufacturer's instructions.

3.5. Immunoassays for *C. difficile* toxins

These assays use polyclonal or monoclonal antibodies against toxin A and/or B. Although most commercial assays now detect both toxin A and B they are relatively insensitive for diagnosis of CDI and are not recommended as standalone tests. However, when combined with a sensitive screening test such as a GDH assay, and/or a NAAT, they provide evidence of *C. difficile* toxin production in vivo (when testing faecal specimens) or in vitro (when testing isolates).

3.5.1 Suitable specimen

Unformed faecal specimens.

3.5.2. Test sensitivity

Reported sensitivities vary with different kits, reference methods used (average values are ~75% and ~50% compared with CCNA and toxigenic culture, respectively)[24, 30, 31]. Sensitivity also varies depending on RT. There is less difference in sensitivity between immunoassays and NAAT when RT027, which produces large amounts of toxin, is present compared to other strains, which produce less - or less antigenic – toxin[2].

3.5.3. Test specificity

Specificities also vary with different kits but are generally >90%.

3.5.4 Predictive values

PPV is reported to be unacceptably low - <50% - for most kits, in populations with a (fairly typical) CDI prevalence of <10%[24]. However, the clinical significance of this is uncertain. A chart review of >6,000 patients with diarrhoea, in whom *C. difficile* toxin tests were performed, showed that only one toxin-negative patient had pseudomembranous colitis and none had other severe CDI complications[39].

3.5.5 Suitable acceptance criteria

Tests should be performed and interpreted according to manufacturer's instructions.

3.6 Combined GDH and toxin assays

Combined GDH and toxin EIAs are also available. They are very rapid, convenient and less expensive than NAAT.

3.6.1. Test sensitivity and specificity.

The sensitivities of the GDH component are similar to those of stand-alone GDH assays and the toxin EIA components have the same limitations as conventional toxin EIAs. Nevertheless, they have the advantage, over NAATs of a similar sensitivity for detection of *C. difficile* and the ability to detect *in vivo* toxin production and thus have generally high specificity for CDI[2].

3.6.2 Predictive values.

Specimens that are GDH and toxin negative or GDH and toxin positive have high NPV and PPV for CDI, respectively. Those that are GDH positive but toxin negative should be tested by a more sensitive method for detection of toxin (e.g. CCNA) or toxin genes (NAAT).

3.7. Nucleic acid amplification tests (NAAT)

In-house NAATs for *C. difficile,* targeting the PaLoc (*tcdA*, *tcdB, tcdE)* and/or 16S rDNA in conventional PCR reactions using gel electrophoresis for product detection, were first described in the 1990s. Despite difficulties with faecal DNA extraction at the time, they were more sensitive than the CCNA and older culture methods, although sometimes less specific because of primer cross-reaction with other *Clostridioides* species. Improved DNA extraction methods have made these assays more practicable and their sensitivity comparable with, and turnaround time much better than, the more sensitive current culture methods[40]. The first commercial NAAT was licensed in 2009 and since then there have been a plethora of assays using many different (usually real time) platforms and product detection methods. Nearly all assays primarily target *tcdB* alone or with one or more additional targets e.g. *tcdC*D117 (to presumptively identify RT 027) and/or CdtLoc (binary toxin); one assay includes a *gyrA* target to detect fluoroquinolone resistance. For assays that do not target *tcdB*, a conserved region of *tcdA* (present as a remnant in *tcdA*-negative/*tcdB*-positive strains) and a combination of *cdtA* and *tcdC*D117 is targeted instead[2, 25]. *C. difficile* is included in several multiplex assays for several viral and bacterial enteropathogens. While this approach is theoretically attractive these assays are expensive and their performance, quality and role in routine diagnosis is yet to be established[25].

3.7.1 Test sensitivity and specificity

When compared with toxigenic culture as gold standard, most NAATs have high analytical sensitivity and specificity (generally <90%); most are fully automated (including extraction) and rapid to perform (1-2 hours) but relatively expensive, compared with immunoassays[2, 25]. However, they have limitations: some toxin A-negative strains(e.g. RT033, among others) are missed by assays that target only *tcdA*[41]; RT 244 – which has recently emerged, in Australia[22] – has the *tcdC*D117 and is misidentified as 027 by assays targeting this deletion[42]. Therefore it is important to recognise that the performance of different assays may vary depending on geographic and temporal difference in RT distribution.

3.7.2 Predictive values

In general the NPV of NAATs is very high. However, even when analytical sensitivities and specificities are optimal, the diagnostic performance is not ideal. Like toxigenic culture, they detect toxigenic *C. difficile* but not necessarily *in vivo* toxin production so their specificities and PPV are poor compared with CCNA and their use may lead to over-diagnosis and inappropriate treatment[8]. The introduction of NAAT into laboratories has resulted in sudden apparent increases in CDI rates, which are potentially misleading in the context of surveillance or public reporting of quality performance data[43, 44]. This over-diagnosis of CDI with PCR has been recognised in clinical settings and has had implications for clinical trials[45].

3.7.3 Cost-effectiveness

Their speed, convenience, sensitivity and specificity have led many laboratories to adopt NAAT as a single diagnostic test for CDI, despite its higher cost compared with immunoassays. A review of diagnostic methods (sponsored by several *C. difficile* NAAT manufacturers) concluded that, despite their greater cost, NAATs were cost-effective as standalone diagnostic tests on the basis of their high sensitivity and rapid turnaround time, which allows more rapid identification and isolation of CDI

patients (or toxigenic *C. difficile* carriers with diarrhoea due to another cause or insignificant diarrhoea) and, hence, reduced risk of transmission[46].

3.7.4 Suitable acceptance criteria

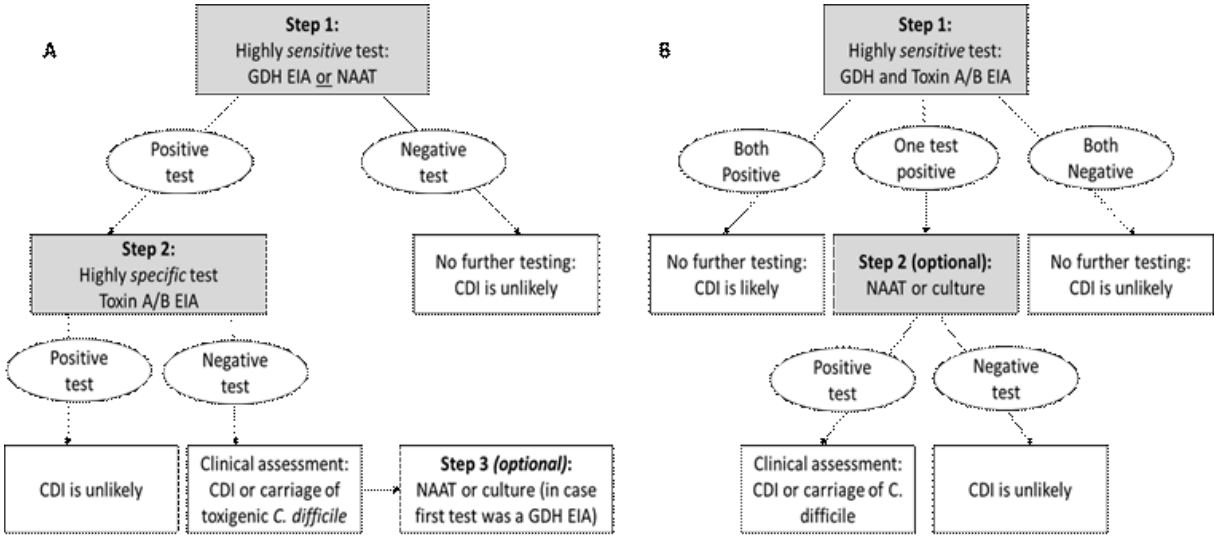
Tests should be performed and interpreted according to manufacturer's instructions.

3.8 Testing algorithms

Several clinical practice guidelines for diagnosis of CDI have been published[29, 31, 34]. Although many laboratories have chosen to use NAATs as a standalone diagnostic test for CDI, others use test combinations, simultaneously or sequentially to optimise clinical predictive values, cost and flexibility, without major increases in turnaround times.

The testing of patients without clinically significant diarrhoea may falsely contribute to a large proportions of the hospital-onset CDI. The consensus protocols between clinicians and diagnostic laboratories at the institutional level are important to optimise utilisation of laboratory resources. Pre-analytical screening has been shown to be an important and effective approach to reduce the burden of inappropriate testing of unformed stools from patients who received laxative therapy[47].

3.8.1 Commonly used CDI testing algorithm:



Notes:

1. GDH and toxin assays can be combined in a single assay. Immunoassays are rapid and easily performed on individual specimens without batching.
2. This algorithm allows all negative and most positive tests to be reported rapidly, with high NPV and PPV and reduces the number of specimens in which NAAT is required to a small proportion of specimens, without loss of sensitivity or significant increase in turnaround time.
3. Clinical records of patients in whom GDH and NAAT are positive, but the toxin immunoassay is negative should be reviewed. These patients may have CDI or could be shedding *C. difficile* in whom diarrhoea is due to another cause.

4. In laboratories in which *C. difficile* cultures are performed to allow strain typing and/or susceptibility testing, cultures can be limited to specimens in which the GDH assay is positive.

4 SNOMED_CT Terminology

SNOMED CT concept	Code
Clostridium difficile (organism)	5933001
Clostridium difficile infection (disorder)	186431008
Clostridium difficile diarrhea (disorder)	5891000119102
Clostridium difficile colitis (disorder)	423590009
Clostridium difficile assay (procedure)	72415005
Clostridium difficile culture (procedure)	122209009
Clostridium difficile toxin assay (procedure)	75332002
Clostridium difficile antigen assay (procedure)	118114008

5 References

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